FATTY ACID EPOXYGENASE GENES FROM PLANTS AND USES THEREFOR IN MODIFYING FATTY ACID METABOLISM

RELATED APPLICATION DATA

This application is a continuation-in-part application of USSN 09/059,769 filed on April 14, 1998, which claims benefit of priority under Title 35, U.S.C. §119 from Australian Patent Application No. PO6223 filed on April 15, 1997 and Australian Patent Application No. PO6226 filed on April 15, 1997, and which also claims benefit of priority under Title 35, U.S.C. §119(e) from USSN 60/043,706 filed on April 16, 1997 and from USSN 60/050,403 filed on June 20, 1997.

FIELD OF THE INVENTION

The present invention relates generally to novel genetic sequences that encode fatty acid epoxygenase enzymes. In particular, the present invention relates to genetic sequences that encode fatty acid Δ12-epoxygenase enzymes as defined herein. More particularly, the present invention provides cDNA and genomic gene sequences that encode plant fatty acid epoxygenases, in particular from *Crepis palaestina* or *Vernonia galamensis*. The genetic sequences of the present invention provide the means by which fatty acid metabolism may be altered or manipulated in organisms such as yeasts, moulds, bacteria, insects, birds, mammals and plants, in particular to convert unsaturated fatty acids to epoxy fatty acids therein. The invention extends to genetically modified oil-accumulating organisms transformed with the subject genetic sequences and to the oils derived therefrom. The oils thus produced provide the means for the cost-effective raw materials for use in the efficient production of coatings, resins, glues, plastics, surfactants and lubricants, amongst others.

GENERAL

Those skilled in the art will be aware that the present invention is subject to variations and modifications other than those specifically described herein. It is to be understood that the invention includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion

of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

This specification contains nucleotide sequence information prepared using the program PatentIn Version 3.1 presented herein after the claims. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier [e.g. <210>1, <210>2, etc]. The length, type of sequence [DNA, protein (PRT), etc] and source organism for each nucleotide sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO: ", followed by the sequence identifier [e.g. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1].

BACKGROUND TO THE INVENTION

There is considerable interest world-wide in producing chemical feedstock, such as fatty acids, for industrial use from renewable plant sources rather than from non-renewable petrochemicals. This concept has broad appeal to manufacturers and consumers on the basis of resource conservation and provides a significant opportunity to develop new industrial crops for agriculture.

There is a diverse array of unusual fatty acids in nature and these have been well characterized (Badam & Patil, 1981; Smith, 1970). Many of these unusual fatty acids have industrial potential and this has led to interest in domesticating such species to enable agricultural production of particular fatty acids.

One class of fatty acids of particular interest are the epoxy-fatty acids, consisting of an acyl chain in which two adjacent carbon bonds are linked by an epoxy bridge. Due to their high reactivity, they have considerable application in the production of coatings, resins, glues, plastics, surfactants and lubricants. These fatty acids are currently produced by chemical epoxidation of vegetable oils, mainly soybean oil and linseed oil, however this process produces mixtures of multiple and isomeric forms and involves significant processing costs.

Attempts are being made by others to develop some wild plants that contain epoxy fatty acids (e.g. *Euphorbia lagascae*, or *Vernonia galamensis*) into commercial sources of these oils. However, problems with agronomic suitability and low yield potential severely limit the commercial utility of traditional plant breeding and cultivation approaches.

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The rapidly increasing sophistication of recombinant DNA technology is greatly facilitating the efficiency of commercially-important industrial processes, by the expression of genes isolated from a first organism or species in a second organism or species to confer novel phenotypes thereon. More particularly, conventional industrial processes can be made

10 more efficient or cost-effective, resulting in greater yields per unit cost by the application of recombinant DNA techniques.

Moreover, the appropriate choice of host organism for the expression of a genetic sequence of interest provides for the production of compounds that are not normally

15 produced or synthesized by the host, at a high yield and purity.

However, despite the general effectiveness of recombinant DNA technology, the isolation of genetic sequences which encode important enzymes in fatty acid metabolism, in particular the genes which encode the fatty acid Δ12-epoxygenase enzymes responsible for

20 producing 12,13-epoxy-9-octadecenoic acid (vernolic acid) and 12,13-epoxy-9,15-octadecadienoic acid, amongst others, remains a major obstacle to the development of genetically-engineered organisms which produce these fatty acids.

Until the present invention, there were only limited biochemical data indicating the nature of

25 fatty acid epoxygenase enzymes, in particular Δ12-epoxygenases. However, in *Euphorbia lagascae*, the formation of 12,13-epoxy-9-octadecenoic acid (vernolic acid) from linoleic acid appears to be catalyzed by a cytochrome-P450-dependent Δ12 epoxygenase enzyme (Bafor *et al.*, 1993; Blee *et al.*, 1994). Additionally, developing seed of linseed plants have the capability to convert added vernolic acid to 12,13-epoxy-9,15-octadecadienoic acid by

30 an endogenous Δ15 desaturase (Engeseth and Stymne, 1996). Epoxy-fatty acids can also be produced by a peroxide-dependent peroxygenase in plant tissues (Blee and Schuber, 1990).

In work leading up to the present invention, the inventors sought to isolate genetic

35 sequences which encode genes which are important for the production of epoxy-fatty acids,

such as 12,13-epoxy-9-octadecenoic acid (vernolic acid) or 12,13-epoxy-9,15-octadecadienoic acid and to transfer these genetic sequences into highly productive commercial oilseed plants and/or other oil accumulating organisms.

5 SUMMARY OF THE INVENTION

One aspect of the invention provides an isolated nucleic acid which encodes or is complementary to an isolated nucleic acid which encodes a fatty acid epoxygenase.

A second aspect of the invention provides an isolated nucleic acid which hybridizes under 10 at least low stringency conditions to at least 20 contiguous nucleotides of SEQ ID NOs:1 or 3 or 5 or 19 or 19, or a complementary sequence thereto.

A further aspect of the invention provides isolated nucleic acid comprising a sequence of nucleotides selected from the group consisting of:

15 (i) a nucleotide sequence that is at least 65% identical to a sequence selected from the group consisting of: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, and SEQ ID NO: 19;

(ii) a nucleotide sequence that encodes an amino acid sequence that is at least about 50% identical to a sequence selected from the group consisting of: SEQ ID NO: 2, 20 SEQ ID NO: 4, SEQ ID NO: 6, and SEQ ID NO: 20; and

(iii) a nucleotide sequence that is complementary to (i) or (ii).

A further aspect of the invention provides a gene construct that comprises the isolated nucleic acid *supra,* in either the sense or antisense orientation, in operable connection with 25 a promoter sequence.

A further aspect of the invention provides a method of altering the level of epoxy fatty acids in a cell, tissue, organ or organism, said method comprising expressing a sense, antisense, ribozyme or co-suppression molecule comprising the isolated nucleic acid *supra* in said cell, 30 tissue, organ or organism for a time and under conditions sufficient for the level of epoxy fatty acids therein to be increased or reduced.

A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising expressing

the isolated nucleic acid *supra* in said cell for a time and under conditions sufficient for the epoxygenase encoded therefor to be produced.

A further aspect of the invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

(i) producing a gene construct which comprises the isolated nucleic acid *supra* placed operably under the control of a promoter capable of conferring expression on said genetic sequence in said cell, and optionally an expression enhancer element;

(ii) transforming said gene construct into said cell; and

(iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level.

A still further aspect of the invention provides a method of producing a recombinant and enzymatically active epoxygenase polypeptide in a transgenic plant comprising the steps of:

(i) producing a gene construct which comprises the isolated nucleic acid *supra* placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said genetic sequences is also placed upstream of a transcription terminator sequence;

(ii) transforming said gene construct into a cell or tissue of said plant; and

(iii) selecting transformants which express a functional epoxygenase encoded by the genetic sequence at a high level in seeds.

A further aspect of the invention provides a recombinant epoxygenase polypeptide or functional enzyme molecule.

A further aspect of the invention provides a recombinant epoxygenase which comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2 or 4 or 6 or 20 or 20 or a homologue, analogue or derivative thereof which is at least about 50% identical thereto. More preferably, the percentage identity to any one of SEQ ID NOs: 2 or 4 or 6 or 20 or 20 is at least about 65%.

A still further aspect of the invention provides a method of producing an epoxy fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or

organism which expresses an enzymatically active recombinant epoxygenase with a fatty acid substrate and preferably, an unsaturated fatty acid substrate, for a time and under conditions sufficient for at least one carbon bond, preferably a carbon double bond, of said substrate to be converted to an epoxy group.

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A further aspect of the invention provides an immunologically interactive molecule which binds to the recombinant epoxygenase polypeptide described herein or a homologue, analogue or derivative thereof.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a linear representation of an expression plasmid comprising an epoxygenase structural gene, placed operably under the control of the truncated napin promoter (FP1; right-hand hatched box) and placed upstream of the NOS terminator sequence (right-hand stippled box). The epoxygenase genetic sequence is indicated by the right-hand open
15 rectangular box. The construct also comprises the NOS promoter (left-hand hatched box) driving expression of the *NPT*II gene (left-hand open box) and placed upstream of the NOS terminator (left-hand stippled box). The left and right border sequences of the *Agrobacterium tumefaciens* Ti plasmid are also indicated.

20 Figure 2 is a schematic representation showing the alignment of the amino acid sequences of the epoxygenase polypeptide of *Crepis palaestina* (Cpa12; SEQ ID NO: 2), a further epoxygenase derived from *Crepis sp.* other than *C. palaestina* which produces high levels of vernolic acid (*CrepX*; SEQ ID NO: 4), a partial amino acid sequence of an epoxygenase polypeptide derived from *Vernonia galamensis* (Vgal1; SEQ ID NO: 6), a full-length amino
25 acid sequence of an epoxygenase polypeptide derived from *Vernonia galamensis* (SEQ ID NO: 20), the amino acid sequence of the Δ12 acetylenase of *Crepis alpina* (Crep1; SEQ ID NO: 8), the Δ12 desaturase of *A. thaliana* (L26296; SEQ ID NO: 9), *Brassica juncea* (X91139; SEQ ID NO: 10), *Glycine max* (L43921; SEQ ID NO: 11), *Solanum commersonii* (X92847; SEQ ID NO: 12) and *Glycine max* (L43920; SEQ ID NO: 13), and the Δ12
30 hydroxylase of *Ricinus communis* (U22378; SEQ ID NO: 14). Underlined are three histidine-rich motifs that are conserved in non-heme containing mixed-function monooxygenases.

Figure 3 is a copy of a photographic representation of a northern blot hybridization showing
35 seed-specific expression of the *Crepis palaestina* epoxygenase gene exemplified by SEQ

ID NO: 1. Northern blot analysis of total RNA from leaves (lane 1) and developing seeds (lane 2) of *Crepis palaestina*. 15µg of total RNA was run on a Northern gel and blotted onto Hybond N^+ membrane from Amersham according to the manufacturer's instructions. The blot was hybridized at 60°C with a probe made from the 3' untranslated region of SEQ ID
5 NO: 1. The blot was washed twice in 2 x SSC (NaCl- Sodium Citrate buffer) at room temperature for 10 minutes, then in 0.1xSSC at 60°C for 20 min.

Figure 4 is a schematic representation of a binary plasmid vector containing an expression cassette comprising the truncated napin seed-specific promoter (Napin) and nopaline
10 synthase terminator (NT), with a *Bam*HI cloning site there between, in addition to the kanamycin-resistance gene *NPT*II operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences.

15 Figure 5 is a schematic representation of a binary plasmid vector containing an expression cassette which comprises SEQ ID NO: 1 placed operably under the control of a truncated napin seed-specific promoter (Napin) and upstream of the nopaline synthase terminator (NT), in addition to the kanamycin-resistance gene *NPT*II operably connected to the nopaline synthase promoter (NP) and nopaline synthase terminator (NT) sequences. The
20 expression cassette is flanked by T-DNA left border (LB) and right-border (RB) sequences. To produce this construct, SEQ ID NO: 1 is inserted into the *Bam*HI site of the binary vector set forth in Figure 4.

Figure 6 is a graphical representation of gas-chromatography traces of fatty acid methyl
25 esters prepared from oil seeds of untransformed *Arabidopsis thaliana* plants [panel (a)], or *A. thaliana* plants (transgenic line Cpal-17) which have been transformed with SEQ ID NO: 1 using the gene construct set forth in Figure 5 [panels (b) and (c)]. In panels (a) and (b), fatty acid methyl esters were separated using packed column separation. In panel (c), the fatty acid methyl esters were separated using capillary column separation. The elution
30 positions of vernolic acid are indicated.

Figure 7 is a graphical representation showing the joint distribution of epoxy fatty acids in selfed seed on T_1 plants of Cpal2-transformed *Arabidopsis thaliana* plants as determined using gas chromatography. Levels of both vernolic acid (x-axis) and 12,13-epoxy-9,15-

octadecadienoic acid (y-axis) were determined and plotted relative to each other. Data show a positive correlation between the levels of these fatty acids in transgenic plants.

Figure 8 is a graphical representation showing the incorporation of ^{14}C-label into the chloroform phase obtained from lipid extraction of linseed cotyledons during labeled-substrate feeding. Symbols used; ♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

Figure 9 is a graphical representation showing the incorporation of ^{14}C-label into the phosphatidyl choline of linseed cotyledons during labeled-substrate feeding. Symbols used; ♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

Figure 10 is a graphical representation showing the incorporation of ^{14}C-label into the triacylglycerols of linseed cotyledons during labeled-substrate feeding. Symbols used♦, [^{14}C] oleic acid feeding; ■, [^{14}C] vernolic acid feeding.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides an isolated nucleic acid which encodes or is complementary to an isolated nucleic acid which encodes a fatty acid epoxygenase.

Wherein the isolated nucleic acid of the invention encodes an enzyme which is involved in the direct epoxidation of arachidonic acid, it is particularly preferred that the subject nucleic acid is derived from a non-mammalian source.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The term "non-mammalian source" refers to any organism other than a mammal or a tissue or cell derived from same. In the present context, the term "derived from a non-mammalian source" shall be taken to indicate that a particular integer or group of integers has been derived from bacteria, yeasts, birds, amphibians, reptiles, insects, plants, fungi, moulds and algae or other non-mammal.

In a preferred embodiment of the present invention, the source organism is any such organism possessing the genetic capacity to synthesize epoxy fatty acids. More preferably,

the source organism is a plant such as, but not limited to *Chrysanthemum spp., Crepis spp., Euphorbia spp.* and *Vernonia spp.,* amongst others.

Even more preferably, the source organism is selected from the group consisting of: *Crepis biennis, Crepis aurea, Crepis conyzaefolia, Crepis intermedia, Crepis occidentalis, Crepis palaestina, Crepis vesicaria, Crepis xacintha, Euphorbia lagascae* and *Vernonia galamensis*. Additional species are not excluded.

In a particularly preferred embodiment of the present invention, the source organism is a *Crepis sp.* comprising high levels of vernolic acid such as *Crepis palaestina,* amongst others or alternatively, *Vernonia galamensis.*

Wherein the isolated nucleic acid of the invention encodes a Δ6-epoxygenase or Δ9-epoxygenase enzyme or Δ12-epoxygenase or Δ15-epoxygenase enzyme, or at least encodes an enzyme which is not involved in the direct epoxidation of arachidonic acid, the subject nucleic acid may be derived from any source producing said enzyme, including, but not limited to, yeasts, moulds, bacteria, insects, birds, mammals and plants.

The nucleic acid of the invention according to any of the foregoing embodiments may be DNA, such as a gene, cDNA molecule, RNA molecule or a synthetic oligonucleotide molecule, whether single-stranded or double-stranded and irrespective of any secondary structure characteristics unless specifically stated.

Reference herein to a "gene" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences);or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred epoxygenase genes of the present invention may be derived from a natural epoxygenase gene by standard recombinant techniques. Generally, an epoxygenase gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions.

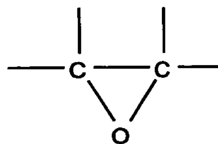
Insertions are those variants in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence, although random insertion is also possible with suitable screening of the resulting product. Nucleotide insertions include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides.

Deletions are variants characterized by the removal of one or more nucleotides from the sequence.

Substitutions are those variants in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, a conservative substitution may alter one amino acid for another similar acting amino acid, or an amino acid of like charge, polarity, or hydrophobicity.

In the context of the present invention, the term "fatty acid epoxygenase" shall be taken to refer to any enzyme or functional equivalent or enzymatically-active derivative thereof that catalyzes the biosynthesis of an epoxy fatty acid, by converting a carbon bond of a fatty acid to an epoxy group and preferably, by converting a carbon double bond of an unsaturated fatty acid to an epoxy group. Although not limiting the invention, a fatty acid epoxygenase may catalyze the biosynthesis of an epoxy fatty acid selected from the group consisting of: (i) 12,13-epoxy-9-octadecenoic acid (vernolic acid); (ii) 12,13-epoxy-9,15-octadecadienoic acid; (iii) 15,16-epoxy-9,12-octadecadienoic acid; (iv) 9,10-epoxy-12-octadecenoic acid; and (v) 9,10-epoxy-octadecanoic acid.

The term "epoxy", or "epoxy group" or "epoxy residue" will be known by those skilled in the art to refer to a three member ring comprising two carbon atoms and an oxygen atom linked by single bonds as follows:



Accordingly, the term "epoxide" refers to a compound that comprise at least one epoxy group as herein before defined.

Those skilled in the art are aware that fatty acid nomenclature is based upon the length of the carbon chain and the position of unsaturated carbon atoms within that carbon chain. Thus, fatty acids are designated using the shorthand notation:

5 $$(\text{Carbon})_{\text{total}}(\text{carbon double bonds})_{\text{total}}{}^{\text{carbon double bond}(\Delta)\text{ position}},$$

wherein the double bonds are *cis* unless otherwise indicated. For example, palmitic acid (*n*-hexadecanoic acid) is a saturated 16-carbon fatty acid (i.e. 16:0), oleic acid (octadecenoic acid) is an unsaturated 18-carbon fatty acid with one double bond between C-9 and C-10 (i.e. $18:1^{\Delta 9}$), and linoleic acid (octadecadienoic acid) is an unsaturated 18-
10 carbon fatty acid with two double bonds between C-9 and C-10 and between C-12 and C-13 (i.e. $18:2^{\Delta 9,12}$).

However, in the present context an epoxygenase enzyme may catalyze the conversion of any carbon bond to an epoxy group or alternatively, the conversion of any double in an
15 unsaturated fatty acid substrate to an epoxy group. In this regard, it is well-known by those skilled in the art that most mono-unsaturated fatty acids of higher organisms are 18-carbon unsaturated fatty acids (i.e. $18:1^{\Delta 9}$), while most polyunsaturated fatty acids derived from higher organisms are 18-carbon fatty acids with at least one of the double bonds therein located between C-9 and C-10. Additionally, bacteria also possess C16- mono-unsaturated
20 fatty acids. Moreover, the epoxygenase of the present invention may act on more than a single fatty acid substrate molecule and, as a consequence, the present invention is not to be limited by the nature of the substrate molecule upon which the subject epoxygenase enzyme acts.

25 Preferably, the substrate molecule for the epoxygenase of the present invention is an unsaturated fatty acid comprising at least one double bond.

Furthermore, epoxygenase enzymes may act upon any number of carbon atoms in any one substrate molecule. For example, they may be characterized as Δ6-epoxygenase, Δ9-
30 epoxygenase, Δ12-epoxygenase or Δ15-epoxygenase enzymes amongst others. Accordingly, the present invention is not limited by the position of the carbon atom in the substrate upon which an epoxygenase enzyme may act.

The term "Δ6-epoxygenase" as used herein shall be taken to refer to an epoxygenase
35 enzyme which catalyzes the conversion of the Δ6 carbon bond of a fatty acid substrate to a

C3 C3 C3 CO F F FU F F C3 F F C3 F

Δ6 epoxy group and preferably, catalyzes the conversion of the Δ6 double bond of at least one unsaturated fatty acid to a Δ6 epoxy group.

The term "Δ9-epoxygenase" as used herein shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ9 carbon bond of a fatty acid substrate to a Δ9 epoxy group and preferably, catalyzes the conversion of the Δ9 double bond of at least one unsaturated fatty acid to a Δ9 epoxy group.

As used herein, the term "Δ12-epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ12 carbon bond of a fatty acid substrate to a Δ12 epoxy group and preferably, catalyzes the conversion of the Δ12 double bond of at least one unsaturated fatty acid to a Δ12 epoxy group.

As used herein, the term "Δ15-epoxygenase" shall be taken to refer to an epoxygenase enzyme which catalyzes the conversion of the Δ15 carbon bond of a fatty acid substrate to a Δ15 epoxy group and preferably, catalyzes the conversion of the Δ15 double bond of at least one unsaturated fatty acid to a Δ15 epoxy group.

The present invention clearly extends to genetic sequences which encode all of the epoxygenase enzymes *listed supra,* amongst others.

In one preferred embodiment of the invention, the isolated nucleic acid encodes a fatty acid epoxygenase enzyme which converts at least one carbon bond in palmitoleic acid ($16:1^{\Delta9}$), oleic acid ($18:1^{\Delta9}$), linoleic acid ($18:2^{\Delta9,12}$), linolenic acid ($18:3^{\Delta9,12,15}$), or arachidonic acid ($20:4^{\Delta5,8,11,14}$) to an epoxy bond. Preferably, the carbon bond is a carbon double bond.

More preferably, the isolated nucleic acid of the invention encodes a fatty acid epoxygenase enzyme that at least converts one or both double bonds in linoleic acid to an epoxy group. According to this embodiment, an epoxygenase which converts both the Δ9 and the Δ12 double bonds of linoleic acid to an epoxy group may catalyze such conversions independently of each other such that said epoxygenase is a Δ9-epoxygenase and/or a Δ12-epoxygenase enzyme as herein before defined.

In an alternative preferred embodiment, the fatty acid epoxygenase of the present invention is a Δ12-epoxygenase, a Δ15- epoxygenase or a Δ9-epoxygenase as herein before defined.

5 More preferably, the fatty acid epoxygenase of the invention is a Δ12- epoxygenase as herein before defined.

In a particularly preferred embodiment of the invention, there is provided an isolated nucleic acid which encodes linoleate Δ12-epoxygenase, the enzyme which at least converts 10 the Δ12 double bond of linoleic acid to a Δ12-epoxy group, thereby producing 12,13-epoxy-9-octadecenoic acid (vernolic acid).

Although not limiting the present invention, the preferred source of the Δ12-epoxygenase of the invention is a plant, in particular *Crepis palaestina* or a further *Crepis sp.* which is 15 distinct from *C. palaestina* but contains high levels of vernolic acid, or *Vernonia galamensis*.

According to this embodiment, a Δ12-epoxygenase may catalyze the conversion of palmitoleic acid to 9,10-epoxy-palmitic acid and/or the conversion of oleic acid to 9,10-epoxy-stearic acid and/or the conversion of linoleic acid to any one or more of 9,10-epoxy-20 12-octadecenoic acid or 12,13-epoxy-9-octadecenoic acid or 9,10,12,13-diepoxy-stearic acid and/or the conversion of linolenic acid to any one or more of 9,10-epoxy-12,15-octadecadienoic acid or 12,13-epoxy-9,15-octadecadienoic acid or 15,16-epoxy-octadecadienoic acid or 9,10,12,13-diepoxy-15-octadecenoic acid or 9,10,15,16-diepoxy-12-octadecenoic acid or 12,13,15,16-diepoxy-9-octadecenoic acid or 9,10,12,13,15,16-25 triepoxy-stearic acid and/or the conversion of arachidonic acid to any one or more of 5,6-epoxy-8,11,14-tetracosatrienoic acid or 8,9-epoxy-5,11,14-tetracosatrienoic acid or 11,12-epoxy-5,8,14-tetracosatrienoic acid or 14,15-epoxy-5,8,11-tetracosatrienoic acid or 5,6,8,9-diepoxy-11,14-tetracosadienoic acid or 5,6,11,12-diepoxy-8,14-tetracosadienoic acid or 5,6,14,15-diepoxy-8,11-tetracosadienoic acid or 8,9,11,12-diepoxy-5,14-tetracosadienoic 30 acid or 8,9,14,15-diepoxy-5,11-tetracosadienoic acid or 11,12,14,15-diepoxy-5,8-tetracosadienoic acid or 5,6,8,9,11,12-triepoxy-14-tetracosenoic acid or 5,6,8,9,14,15-triepoxy-11-tetracosenoic acid or 5,6,11,12,14,15-triepoxy-8-tetracosenoic acid or 8,9,11,12,14,15-triepoxy-5-tetracosenoic acid, amongst others.

Those skilled in the art may be aware that not all substrates listed *supra* may be derivable from a natural source, but notwithstanding this, may be produced by chemical synthetic means. The conversion of both natural and synthetic unsaturated fatty acids to epoxy fatty acids is clearly within the scope of the present invention.

The present invention is particularly directed to those epoxygenase enzymes that are mixed-function monooxygenase enzymes, and nucleic acids encoding said enzymes, and uses of said enzymes and nucleic acids. Accordingly, it is particularly preferred that the nucleic acid of the invention encode a fatty acid epoxygenase which is a mixed-function monooxygenase enzyme.

In the context of the present invention, the term "mixed-function monooxygenase enzyme" shall be taken to refer to any epoxygenase polypeptide that comprises an amino acid sequence comprising three histidine-rich regions as follows:

(i) His-$(Xaa)_{3-4}$-His (SEQ ID NO: 21 and SEQ ID NO: 22);

(ii) His-$(Xaa)_{2-3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24); and

(iii) His-$(Xaa)_{2-3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24),

wherein His designates histidine, Xaa designates any naturally-occurring amino acid residue as set forth in Table 1 herein, the integer $(Xaa)_{3-4}$ refers to a sequence of amino acids comprising three or four repeats of Xaa, and the integer $(Xaa)_{2-3}$ refers to a sequence of amino acids comprising two or three repeats of Xaa.

In the exemplification of the invention described herein, the inventors provide isolated cDNAs that comprise nucleotide sequences encoding the Δ12-epoxygenase polypeptides of *Crepis palaestina* and *Vernonia galamensis*. Each exemplified full-length amino acid sequence encoded by said cDNAs which includes the three characteristic amino acid sequence motifs of a mixed-function monooxygenase enzyme as herein before defined. Close sequence identity between the amino acid sequences of the Δ12-epoxygenase enzymes from *C. palaestina* (SEQ ID NO: 2), an unidentified *Crepis sp* (SEQ ID NO: 4), and *Vernonia galamensis* (SEQ ID NO: 20), suggests functional similarity between these polypeptides. In contrast, the amino acid sequences of these epoxygenases have lower identity to the amino acid sequences of a fatty acid desaturase or a fatty acid hydroxylase.

It is even more preferred that the epoxygenase of the present invention at least comprises a sequence of amino acids which comprises three histidine-rich regions as follows:

(i) His-Glu-Cys-Gly-His-His (SEQ ID NO: 15);

(ii) His-Arg-Asn-His-His (SEQ ID NO: 16); and

(iii) His-Val-Met-His-His (SEQ ID NO: 17) or His-Val-Leu-His-His (SEQ ID NO: 18),

5 wherein His designates histidine, Glu designates glutamate, Cys designates cysteine, Gly designates glycine, Arg designates arginine, Asn designates asparagine, Val designates valine, Met designates methionine and Leu designates leucine.

The present invention clearly extends to epoxygenase genes derived from other species, 10 including the epoxygenase genes derived from *Chrysanthemum spp.* and *Euphorbia lagascae*, amongst others.

In a preferred embodiment, whilst not limiting the present invention, the epoxygenase genes of other species which are encompassed by the present invention encode mixed-15 function monooxygenase enzymes. The present invention further extends to the isolated or recombinant polypeptides encoded by such genes and uses of said genes and polypeptides.

The invention described according to this embodiment does not encompass nucleic acids 20 which encode enzyme activities other than epoxygenase activities as defined herein, in particular the Δ12-desaturase enzymes derived from *Arabidopsis thaliana, Brassica juncea, Brassica napus* or *Glycine max,* amongst others, which are known to contain similar histidine-rich motifs.

25 In the present context, "homologues" of an amino acid sequence refer to those amino acid sequences or peptide sequences which are derived from polypeptides, enzymes or proteins of the present invention or alternatively, correspond substantially to the amino acid sequences listed *supra*, notwithstanding any naturally-occurring amino acid substitutions, additions or deletions thereto.

30

For example, amino acids may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on. Alternatively, or in addition, the amino acids of a homologous amino acid sequence may be replaced by other

amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

Naturally-occurring amino acid residues contemplated herein are described in Table 1.

A homologue of an amino acid sequence may be a synthetic peptide produced by any method known to those skilled in the art, such as by using Fmoc chemistry.

Alternatively, a homologue of an amino acid sequence may be derived from a natural source, such as the same or another species as the polypeptides, enzymes or proteins of the present invention. Preferred sources of homologues of the amino acid sequences listed *supra* include any of the sources contemplated herein.

"Analogues" of an amino acid sequence encompass those amino acid sequences which are substantially identical to the amino acid sequences listed *supra* notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

Preferred non-naturally occurring amino acids contemplated herein are listed below in Table 2.

The term "derivative" in relation to an amino acid sequence shall be taken to refer hereinafter to mutants, parts, fragments or polypeptide fusions of the amino acid sequences listed *supra*. Derivatives include modified amino acid sequences or peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are also contemplated by the present invention. Additionally, derivatives may comprise fragments or parts of an amino acid sequence disclosed herein and are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject sequences.

Procedures for derivatizing peptides are well-known in the art.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions

may be classified as "conservative", in which case an amino acid residue is replaced with another naturally-occurring amino acid of similar character, for example Gly⇔Ala, Val⇔Ile⇔Leu, Asp⇔Glu, Lys⇔Arg, Asn⇔Gln or Phe⇔Trp⇔Tyr.

5 Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in a repressor polypeptide is substituted with an amino acid having different properties, such as a naturally-occurring amino acid from a different group (e.g. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-
10 conventional amino acid.

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

15 Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

20

The present invention clearly extends to the subject isolated nucleic acid when integrated into the genome of a cell as an addition to the endogenous cellular complement of epoxygenase genes. Alternatively, wherein the host cell does not normally encode enzymes required for epoxy fatty acid biosynthesis, the present invention extends to the
25 subject isolated nucleic acid when integrated into the genome of said cell as an addition to the endogenous cellular genome.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen

	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
5	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
10	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
15	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
20	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl) glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl) glycine	Nbhe
25	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl) glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
30	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)) glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
35	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen

N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomo phenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl) glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomo phenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine		N-(N-(3,3-diphenylpropyl) Nnbhmcarbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

A second aspect of the present invention provides an isolated nucleic acid which comprises the sequence of nucleotides set forth in any one of SEQ ID NOs:1 or 3 or 5 or 19or a complementary sequence thereto, or a homologue, analogue or derivative thereof.

5 For the purposes of nomenclature, the nucleotide sequence set forth in SEQ ID NO: 1 is derived from *Crepis palaestina* and encodes the mixed function monooxygenase sequence or mixed function monooxygenase-like sequence set forth in SEQ ID NO: 2. As exemplified herein, the amino acid sequence set forth in SEQ ID NO: 2 has epoxygenase activity, more particularly Δ12-epoxygenase activity.

10

The nucleotide sequence set forth in SEQ ID NO: 3 corresponds to a cDNA derived from a *Crepis sp.* other than *C. palaestina* comprising high levels of vernolic acid. The amino acid sequence set forth in SEQ ID NO: 4 corresponds to the derived amino acid sequence of the *Crepis sp.* epoxygenase gene provided in SEQ ID NO: 3.

15

The nucleotide sequence set forth in SEQ ID NO: 5 corresponds to amplified DNA derived from *Vernonia galamensis* using amplification primers derived from a consensus sequence of mixed function monooxygenases, including the *Crepis spp.* epoxygenase gene sequences of the invention. The amplified DNA comprises a partial epoxygenase gene
20 sequence, which includes nucleotide sequences capable of encoding the histidine-rich motif His-Arg-Asn-His-His which is characteristic of mixed function monooxygenase enzymes. The amino acid sequence set forth in SEQ ID NO: 6 corresponds to the derived amino acid sequence of the *Vernonia galamensis* epoxygenase gene provided in SEQ ID NO: 5.

25 The nucleotide sequence set forth in SEQ ID NO: 19 derived from *Vernonia galamensis* and encodes the full-length mixed function monooxygenase set forth in SEQ ID NO: 20.

The nucleotide sequence set forth in SEQ ID NO: 7 relates to the partial sequence of a *Crepis alpina* acetylenase gene which was used as a probe to isolate the nucleic acid
30 comprising the nucleotide sequence set forth in SEQ ID NO: 1. The amino acid sequence set forth in SEQ ID NO: 8 corresponds to the derived amino acid sequence of said partial sequence of the *C. alpina* acetylenase gene.

As used herein, the term "acetylenase" shall be taken to refer to an enzyme which is
35 capable of catalyzing the conversion of a carbon double bond in a fatty acid substrate

molecule to a carbon triple bond or alternatively, which is capable of catalyzing the formation of a carbon triple bond in a fatty acid molecule.

The present invention clearly extends to the genomic gene equivalents of the cDNA
5 molecules exemplified in any one of SEQ ID NOs: 1, 3, 5, or 19.

In a most particularly preferred embodiment, the present invention provides an isolated nucleic acid which comprises the nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 5, or 19 or a genomic gene equivalent of said nucleotide sequence or a homologue,
10 analogue or derivative thereof.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid which is substantially the same as the nucleic acid of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within
15 said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid which is substantially the same as a nucleic acid of the present invention or its
20 complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

25 "Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid comprising significant sequence similarity to said sequence or a part thereof.

Generally, homologues, analogues or derivatives of the nucleic acid of the invention are
30 produced by synthetic means or alternatively, derived from naturally-occurring sources. For example, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions as indicated *supra*.

In one embodiment of the invention, preferred homologues, analogues or derivatives of the nucleotide sequences set forth in any one of SEQ ID NOs: 1, 3, 5, or 19 or complementary sequences thereto, encode immunologically-active or enzymatically-active polypeptides.

5 As used herein, the term "immunologically-active" shall be taken to refer to the ability of a polypeptide molecule to elicit an immune response in a mammal, in particular an immune response sufficient to produce an antibody molecule such as, but not limited to, an IgM or IgG molecule or whole serum containing said antibody molecule. The term "immunologically-active" also extends to the ability of a polypeptide to elicit a sufficient 10 immune response for the production of monoclonal antibodies, synthetic Fab fragments of an antibody molecule, single-chain antibody molecule or other immunointeractive molecule.

As used herein, the term "enzymatically-active" shall be taken to refer to the ability of a polypeptide molecule to catalyze an enzyme reaction, in particular an enzyme reaction 15 which comprises the epoxygenation of a carbon bond in a fatty acid substrate molecule. More particularly, whilst not limiting the invention, the term "enzymatically-active" may also refer to the ability of a polypeptide molecule to catalyze the epoxygenation of Δ-9 or Δ-12 in a fatty acid substrate molecule such as linoleic acid or vernolic acid.

20 In an alternative embodiment, a preferred homologue, analogue or derivative of the nucleotide sequence set forth in any one of SEQ ID NOs: 1 or 3 or 5 or 19, or a complementary sequence thereto, comprises a sequence of nucleotides which is at least 65% identical to at least 20 contiguous nucleotides therein, other than a nucleotide sequence which encodes a *Crepis sp.* acetylenase enzyme.

25

More preferably, the percentage identity to any one of SEQ ID NOs: 1 or 3 or 5 or 19 is at least about 85%. Even more preferably, a homologue, analogue or derivative of SEQ ID NOs: 1 or 3 or 5 or 19 is at least about 90% and even more preferably at least about 95% identical to at least 100 or 250 or 500 or 1000 contiguous nucleotides therein.

30

Reference herein to a percentage identity or percentage similarity between two or more nucleotide or amino acid sequences shall be taken to refer to the number of identical or similar residues in a nucleotide or amino acid sequence alignment, as determined using any standard algorithm known by those skilled in the art. In particular, nucleotide and/or 35 amino acid sequence identities and similarities may be calculated using the Gap program,

which utilizes the algorithm of Needleman and Wunsch (1970) to maximize the number of residue matches and minimize the number of sequence gaps. The Gap program is part of the Sequence and Analysis Software Package of the Computer Genetics Group Inc., University Research Park, Madison, Wisconsin, United States of America (Devereux *et al.*, 1984).

In a further alternative embodiment, a preferred homologue, analogue or derivative of the nucleotide sequence set forth in any one of SEQ ID NOs: 1, 3, 5, or 19 or a complementary sequence thereto, hybridizes under at least low stringency conditions to at least 20 contiguous nucleotides derived from said sequence.

More preferably, the stringency of hybridization is at least moderate stringency, even more preferably at least high stringency.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridization conditions may be employed. For example, a low stringency may comprise a hybridization and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. A moderate stringency may comprise a hybridization and/or wash carried out in 2 x SSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency may comprise a hybridization and/or wash carried out in 0.1 x SSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in the hybridization buffer or wash buffer and/or increasing the temperature at which the hybridization and/or wash are performed. Conditions for a hybridization and/or wash are well understood by one normally skilled in the art. For the purposes of clarification of parameters affecting hybridization between nucleic acids, reference can conveniently be made to pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

The isolated nucleic acids disclosed herein may be used to isolate or identify homologues, analogues or derivatives thereof from other cells, tissues, or organ types, or from the cells, tissues, or organs of another species using any one of a number of means known to those skilled in the art.

For example, genomic DNA, or mRNA, or cDNA may be contacted, under at least low stringency hybridization conditions or equivalent, with a hybridization effective amount of an isolated nucleic acid which comprises the nucleotide sequence set forth in any one SEQ ID NOs: 1, 3, 5, or 19 or a complementary sequence thereto, or a functional part thereof, and the hybridization detected using a detection means.

The detection means may be a reporter molecule capable of giving an identifiable signal (e.g. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule) covalently linked to the isolated nucleic acid of the invention.

In an alternative method, the detection means is any known format of the polymerase chain reaction (PCR). According to this method, degenerate pools of nucleic acid "primer molecules" of about 15-50 nucleotides in length are designed based upon the nucleotide sequences disclosed in SEQ ID NOs: 1, 3, 5, or 19 or a complementary sequence thereto. The homologues, analogues or derivatives (i.e. the "template molecule") are hybridized to two of said primer molecules, such that a first primer hybridizes to a region on one strand of the template molecule and a second primer hybridizes to a complementary sequence thereof, wherein the first and second primers are not hybridized within the same or overlapping regions of the template molecule and wherein each primer is positioned in a 5'- to 3'- orientation relative to the position at which the other primer is hybridized on the opposite strand. Specific nucleic acid copies of the template molecule are amplified enzymatically in a polymerase chain reaction, a technique that is well known to one skilled in the art.

The primer molecules may comprise any naturally-occurring nucleotide residue (i.e. adenine, cytidine, guanine, thymidine) and/or comprise inosine or functional analogues or derivatives thereof, capable of being incorporated into a polynucleotide molecule. The nucleic acid primer molecules may also be contained in an aqueous mixture of other nucleic acid primer molecules or be in a substantially pure form.

The detected sequence may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related genetic sequence originates from another plant species.

A third aspect of the present invention provides an isolated nucleic acid which encodes the amino acid sequence set forth in any one of SEQ ID NOs: 2 or 4 or 6 or 20 or a homologue, analogue or derivative thereof.

5 In one embodiment contemplated herein, preferred homologues, analogues or derivatives of the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, or 20 are immunologically-active or enzymatically-active polypeptides as defined *supra*.

In an alternative embodiment of the invention, preferred homologues, analogues or 10 derivatives of the amino acid sequence set forth in any one of SEQ ID NOs: 2, 4, 6, or 20 comprise a sequence of amino acids which is at least 65% identical thereto, other than a *Crepis sp.* acetylenase polypeptide. More preferably, homologues, analogues or derivatives of SEQ ID NOs: 2 or 4 or 6 or 20 which are encompassed by the present invention are at least about 85% identical, even more preferably at least about 90% 15 identical and still even more preferably at least about 95% identical, and still more preferably at least about 99%-100% identical thereto.

Homologues, analogues or derivatives of any one of SEQ ID NOs: 2 or 4 or 6 or 20 may further comprise a histidine-rich region as defined *supra*. Even more preferably, the subject 20 epoxygenase at least comprises a sequence of amino acids which comprises three histidine rich regions as follows:

(i) His-Glu-Cys-Gly-His-His (SEQ ID NO: 15);

(ii) His-Arg-Asn-His-His (SEQ ID NO: 16); and

(iii) His-Val-Met-His-His (SEQ ID NO: 17) or His-Val-Leu-His-His (SEQ 25 ID NO: 18),

or a homologue, analogue or derivative thereof.

The invention described according to this alternative embodiment does not encompass the Δ12-desaturase enzymes derived from *Arabidopsis thaliana, Brassica juncea, Brassica* 30 *napus* or *Glycine max,* amongst others.

The isolated nucleic acid of the present invention is useful for developing gene constructs comprising a sense molecule wherein said gene constructs are designed for the expression in a cell which does not normally express said nucleic acid or over-expression of said 35 nucleic acid in a cell which does normally express the said nucleic acid.

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Accordingly, a further aspect of the invention provides a gene construct which comprises a sense molecule which is operably connected to a promoter sequence.

5 The term "sense molecule" as used herein shall be taken to refer to an isolated nucleic acid which encodes or is complementary to an isolated nucleic acid which encodes a fatty acid epoxygenase wherein said nucleic acid is provided in a format suitable for its expression to produce a recombinant polypeptide when said sense molecule is introduced into a host cell by transfection or transformation.

10

Those skilled in the art will be aware that a gene construct may be used to "transfect" a cell, in which case it is introduced into said cell without integration into the cell's genome. Alternatively, a gene construct may be used to "transform" a cell, in which case it is stably integrated into the genome of said cell.

15

A sense molecule that comprises a fatty acid epoxygenase gene sequence or homologue, analogue or derivative thereof, may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed 20 cell, using any method known to those skilled in the art.

Thus, the epoxygenase genes described herein may be used to develop single cells or whole organisms which synthesize epoxy fatty acids not normally produced by wild or naturally-occurring organisms belonging to the same genera or species as the genera or 25 species from which the transfected or transformed cell is derived, or to increase the levels of such fatty acids above the levels normally found in such wild or naturally-occurring organisms.

In an alternative preferred embodiment, the isolated nucleic acid of the invention is capable 30 of reducing the level of epoxy fatty acids in a cell, when expressed therein, in the antisense orientation or as a ribozyme or co-suppression molecule, under the control of a suitable promoter sequence.

Co-suppression is the reduction in expression of an endogenous gene that occurs when 35 one or more copies of said gene, or one or more copies of a substantially similar gene are

introduced into the cell. The present invention also extends to the use of co-suppression to inhibit the expression of an epoxygenase gene as described herein.

In the context of the present invention, an antisense molecule is an RNA molecule which is
5 transcribed from the complementary strand of a nuclear gene to that which is normally transcribed to produce a "sense" mRNA molecule capable of being translated into a polypeptide. The antisense molecule is therefore complementary to the sense mRNA, or a part thereof. Although not limiting the mode of action of the antisense molecules of the present invention to any specific mechanism, the antisense RNA molecule possesses the
10 capacity to form a double-stranded mRNA by base pairing with the sense mRNA, which may prevent translation of the sense mRNA and subsequent synthesis of a polypeptide gene product.

Ribozymes are synthetic RNA molecules which comprise a hybridizing region
15 complementary to two regions, each of at least 5 contiguous nucleotide bases in the target sense mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA. A complete description of the function of ribozymes is presented by Haseloff and Gerlach (1988) and contained in International Patent Application No. WO89/05852. The present invention extends to
20 ribozymes which target a sense mRNA encoding an epoxygenase polypeptide described herein, thereby hybridizing to said sense mRNA and cleaving it, such that it is no longer capable of being translated to synthesize a functional polypeptide product.

According to this embodiment, the present invention provides a ribozyme or antisense
25 molecule comprising a sequence of contiguous nucleotide bases which are able to form a hydrogen-bonded complex with a sense mRNA encoding an epoxygenase described herein, to reduce translation of said mRNA. Although the preferred antisense and/or ribozyme molecules hybridize to at least about 10 to 20 nucleotides of the target molecule, the present invention extends to molecules capable of hybridizing to at least about 50-100
30 nucleotide bases in length, or a molecule capable of hybridizing to a full-length or substantially full-length epoxygenase mRNA.

It is understood in the art that certain modifications, including nucleotide substitutions amongst others, may be made to the antisense and/or ribozyme molecules of the present
35 invention, without destroying the efficacy of said molecules in inhibiting the expression of

the epoxygenase gene. It is therefore within the scope of the present invention to include any nucleotide sequence variants, homologues, analogues, or fragments of the said gene encoding same, the only requirement being that said nucleotide sequence variant, when transcribed, produces an antisense and/or ribozyme molecule which is capable of hybridizing to the said sense mRNA molecule.

The present invention extends to gene constructs designed to facilitate expression of a sense molecule, an antisense molecule, ribozyme molecule, or co-suppression molecule which is capable of altering the level of epoxy fatty acids in a cell.

In a particularly preferred embodiment, the sense molecule, an antisense molecule, ribozyme molecule, co-suppression molecule, or gene targeting molecule which is capable of altering the epoxy fatty acid composition of a cell derived from plant or other organism comprises a sequence of nucleotides set forth in any one of SEQ ID NOs: 1, 3, 5, or 19 or a complementary strand, homologue, analogue or derivative thereof.

Those skilled in the art will also be aware that expression of a sense, antisense, ribozyme or co-suppression molecule may require the nucleic acid of the invention to be placed in operable connection with a promoter sequence. The choice of promoter for the present purpose may vary depending upon the level of expression of the sense molecule required and/or the species from which the host cell is derived and/or the tissue-specificity or development-specificity of expression of the sense molecule which is required.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. In the context of the present invention, the term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of said sense

molecule in a cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, copper-responsive regulatory elements may be placed adjacent to a heterologous
5 promoter sequence driving expression of a sense molecule to confer copper inducible expression thereon.

Placing a sense, antisense, ribozyme or co-suppression molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is
10 controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream or 5' of a nucleic acid which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the sense, antisense, ribozyme or co-suppression molecule or chimeric gene comprising same. In the construction of heterologous promoter/structural gene combinations it is
15 generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect
20 to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include
25 promoters derived from the genes of viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants which are capable of functioning in isolated cells or whole organisms regenerated therefrom. The promoter may regulate the expression of the sense, antisense, ribozyme or co-suppression molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which
30 expression occurs, or in response to external stimuli such as physiological stresses, pathogens, or metal ions, amongst others.

Examples of promoters include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, *Arabidopsis thaliana SSU* gene promoter, napin seed-specific promoter,
35 P_{32} promoter, BK5-T *imm* promoter, *lac* promoter, *tac* promoter, phage lambda λ_L or λ_R

promoters, CMV promoter (U.S. Patent No. 5,168,062), T7 promoter, lacUV5 promoter, SV40 early promoter (U.S. Patent No. 5,118,627), SV40 late promoter (U.S. Patent No. 5,118,627), adenovirus promoter, baculovirus P10 or polyhedrin promoter (U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784), and the like. In addition to
5 the specific promoters identified herein, cellular promoters for so-called housekeeping genes are useful.

Preferred promoters according to this embodiment are those promoters which are capable of functioning in yeast, mould or plant cells. More preferably, promoters suitable for use
10 according to this embodiment are capable of functioning in cells derived from oleaginous yeasts, oleaginous moulds or oilseed crop plants, such as flax sold under the trademark Linola™ (hereinafter referred to as "Linola™ flax"), sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

15 In a more preferred embodiment, the promoter may be derived from a genomic clone encoding an epoxygenase enzyme, preferably derived from the genomic gene equivalents of epoxygenase genes derived from *Chrysanthemum spp., Crepis spp.* including *C. palaestina* or other *Crepis sp.*, *Euphorbia lagascae* or *Vernonia galamensis,* which are referred to herein.

20

In a more preferred embodiment, the promoter may be derived from a highly-expressed seed gene, such as the napin gene, amongst others.

The gene construct of the invention may further comprise a terminator sequence and be
25 introduced into a suitable host cell where it is capable of being expressed to produce a recombinant polypeptide gene product or alternatively, a ribozyme or antisense molecule.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences
30 containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

35

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens,* the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E. coli* terminator, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (e.g. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene and luciferase gene, amongst others.

A further aspect of the present invention provides a transfected or transformed cell, tissue, organ or whole organism which expresses a recombinant epoxygenase polypeptide or a

ribozyme, antisense or co-suppression molecule as described herein, or a homologue, analogue or derivative thereof.

Preferably, the isolated nucleic acid is contained within a gene construct as described herein. The gene construct of the present invention may be introduced into a cell by various techniques known to those skilled in the art. The technique used may vary depending on the known successful techniques for that particular organism.

Means for introducing recombinant DNA into bacterial cells, yeast cells, or plant, insect, fungal (including mould), avian or mammalian tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.,* 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

In a particularly preferred embodiment, wherein the gene construct comprises a "sense" molecule, it is particularly preferred that the recombinant epoxygenase polypeptide produced therefrom is enzymatically active.

Alternatively, wherein the cell is derived from a multicellular organism and where relevant technology is available, a whole organism may be regenerated from the transformed cell, in accordance with procedures well known in the art.

5 Those skilled in the art will also be aware of the methods for transforming, regenerating and propagating other type of cells, such as those of fungi.

In the case of plants, plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present 10 invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue 15 (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers.

20 The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The regenerated transformed plants may be propagated by a variety of means, such as by 25 clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The regenerated transformed organisms contemplated herein may take a variety of forms. 30 For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

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A further aspect of the invention provides a method of altering the level of epoxy fatty acids in a cell, tissue, organ or organism, said method comprising expressing a sense, antisense, ribozyme or co-suppression molecule as described herein in said cell for a time and under conditions sufficient for the level of epoxy fatty acids therein to be increased or reduced.

5

In a preferred embodiment, the subject method comprises the additional first step of transforming the cell, tissue, organ or organism with the sense, antisense, ribozyme or co-suppression molecule.

10 As discussed *supra* the isolated nucleic acid may be contained within a gene construct.

According to this embodiment, the cell, organ, tissue or organism in which the subject sense, antisense, ribozyme or co-suppression molecule is expressed may be derived from a bacteria, yeast, fungus (including a mould), insect, plant, bird or mammal.

15

Because a recombinant epoxygenase polypeptide may be produced in the regenerated transformant as well as *ex vivo*, one alternative preferred embodiment of the present invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a cell, said method comprising the steps of:

20 (i) producing a gene construct which comprises the cDNA or genomic epoxygenase genetic sequence of the invention placed operably under the control of a promoter capable of conferring expression on said genetic sequence in said cell, and optionally an expression enhancer element;

(ii) transforming said gene construct into said cell; and

25 (iii) selecting transformants which express the epoxygenase encoded by the genetic sequence at a high level.

A particularly preferred embodiment of the present invention provides a method of producing a recombinant enzymatically active epoxygenase polypeptide in a transgenic

30 plant comprising the steps of:

(i) producing a gene construct which comprises the cDNA or genomic epoxygenase genetic sequence of the invention placed operably under the control of a seed-specific promoter and optionally an expression enhancer element, wherein said genetic sequences is also placed upstream of a

35 transcription terminator sequence;

(ii) transforming said gene construct into a cell or tissue of said plant; and

(iii) selecting transformants which express the epoxygenase encoded by the genetic sequence at a high level in seeds.

5 In a more particularly preferred embodiment, the plant is an oilseed species that normally produces significant levels of linoleic acid, for example Linola™ flax, oilseed rape, sunflower, safflower, soybean, linseed, sesame, cottonseed, peanut, olive or oil palm, amongst others.

10 In an even more particularly preferred embodiment, the plant is an oilseed species that normally produces significant levels of linoleic acid, for example Linola™ flax, sunflower or safflower, amongst others.

Enzymatically active recombinant epoxygenases described herein are particularly useful for

15 the production of epoxy fatty acids from unsaturated fatty acid substrates. The present invention especially contemplates the production of specific epoxy fatty acids in cells or regenerated transformed organisms which do not normally produce that specific epoxy fatty acid.

20 Accordingly, a further aspect of the invention provides a method of producing an epoxy fatty acid in a cell, tissue, organ or organism, said method comprising incubating a cell, tissue, organ or organism which expresses an enzymatically active recombinant epoxygenase of the present invention with a fatty acid substrate molecule, preferably an unsaturated fatty acid substrate molecule, for a time and under conditions sufficient for at least one carbon

25 bond of said substrate to be converted to an epoxy group.

In an alternative embodiment, the subject method further comprises the additional first step of transforming or transfecting the cell, tissue, organ or organism with a nucleic acid which encodes said recombinant epoxygenase or a homologue, analogue or derivative thereof, as

30 herein before described. As discussed *supra* the isolated nucleic acid may be contained within a gene construct.

According to this embodiment, the cell, organ, tissue or organism in which the subject epoxygenase is expressed is derived from a bacteria, yeast, fungus (including a mould),

35 insect, plant, bird or mammal. More preferably, the cell, organ, tissue or organism is derived

from a yeast, plant or fungus, even more preferably from an oleaginous yeast or plant or fungus, or from an oilseed plant which does not normally express the recombinant epoxygenase of the invention.

5 Amongst the main economic oilseed plants contemplated herein, high-linoleic genotypes of flax, sunflower, corn and safflower are preferred targets. Soybean and rape seed are alternative targets but are less suitable for maximal epoxy fatty acid synthesis because of their lower levels of linoleic acid substrate and the presence of an active Δ15-desaturase competing with the epoxygenase for the linoleic acid substrate.

10

An alternative embodiment is the transformation of Linola™ (= low linolenic acid flax) with the epoxygenase of the invention. Linola™ flax normally contains around 70% linoleic acid with very little of this (<2%) being subsequently converted to linolenic acid by Δ15-desaturase (Green, 1986).

15

Preferred unsaturated fatty acid substrates contemplated herein include, but are not limited to, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid, amongst others.

20 In plant species that naturally contain high levels of vernolic acid, the Δ12-epoxygenase therein may be very efficient at carryong out the epoxidation of linoleic acid. As a consequence, the present invention particularly contemplates the expression of recombinant Δ12-epoxygenase derived from *Euphorbia lagascae*, *Vernonia spp.* and *Crepis spp.* at high levels in transgenic oilseeds during seed oil synthesis, to produce high

25 levels of vernolic acid therein.

Accordingly, linoleic acid is a particularly preferred substrate according to this embodiment of the invention. Additional substrates are not excluded.

30 The products of the substrate molecules listed *supra* will be readily determined by those skilled in the art, without undue experimentation. Particularly preferred epoxy fatty acids produced according to the present invention include 12,13-epoxy-9-octadecenoic acid (vernolic acid) and 12,13-epoxy-9,15-octadecadienoic acid, amongst others.

Conditions for the incubation of cells, organs, tissues or organisms expressing the recombinant epoxygenase in the presence of the substrate molecule will vary, at least depending upon the uptake of the substrate into the cell, tissue, organ or organism, and the affinity of the epoxygenase for the substrate molecule in the particular environment
5 selected. Optimum conditions may be readily determined by those skilled in the relevant art.

The present invention clearly extends to the isolated oil containing epoxy fatty acids, and/or the isolated epoxy fatty acid itself produced as described herein and to any products derived therefrom, for example coatings, resins, glues, plastics, surfactants and lubricants,
10 amongst others.

The inventors have shown further that the mixed function monooxygenases (MMO) which perform catalytic functions such as desaturation, acetylenation, hydroxylation and/or epoxygenation, form a family of genes sharing considerable nucleotide and amino acid
15 sequence similarity. For example, the desaturase, acetylenase, hydroxylase and/or epoxygenase enzymes which act on substrate molecules having a similar chain length and position of any carbon double bond(s) (if present) are more closely related to each other than to enzymes acting upon other substrates, and may be considered to be a "family".

20 Without being bound by any theory or mode of action, the sequence similarity between the members of any gene family has its basis in the identity of the substrate involved and the biochemical similarity of the reaction events occurring at the target carbon bond during the modification reaction, suggesting that divergent sequences within a family may comprise catalytic determinants or at least a functional part thereof which contributes to the specific
25 catalytic properties of the family members.

One example of a family is the desaturase, acetylenase, hydroxylase and/or epoxygenase enzymes which catalyze desaturation, acetylenation, hydroxylation and/or epoxygenation respectively, of the Δ12 position of linoleic acid (hereinafter referred to as the "C18 Δ12-
30 MMO family"). The present inventors have compared the nucleotide and amino acid sequences of members of the C18 Δ12-MMO family to determine the divergent regions thereof which potentially comprise the determinants of alternative catalytic functions at the Δ12 position (hereinafter referred to as "putative catalytic determinants").

Furthermore, the presence of such families of fatty acid modifying MMOs is contemplated with respect to other fatty acid chain length and double bond positions. For example, the C18 Δ15-desaturase is contemplated to belong to a family of related enzymes capable of desaturation, acetylenation, hydroxylation and/or epoxidation of the Δ15 position in C18 fatty acid substrates, the C18 Δ15-MMO family.

By producing synthetic genes in which these catalytic determinants have been interchanged (referred to as "domain swapping") it is possible to convert genes encoding one catalytic function into those encoding alternative catalytic functions. For example, the Δ12 epoxygenase of the instant invention may be converted to a Δ12 acetylenase by replacing portions of its C-terminal and N-terminal sequences with the equivalent domains from the *Crepis alpina* Δ12 acetylenase. Similarly, the reverse domain swapping may also be performed.

As a further refinement, such changes in catalytic function can similarly be effected by making specific changes (e.g. addition, substitution or deletion) to only those amino-acids within each domain that are critical for determining the relevant catalytic function (such as by site-directed mutagenesis).

Accordingly, a further aspect of the present invention contemplates a synthetic fatty acid gene comprising a sequence of nucleotides derived from an epoxygenase gene as described herein, wherein said synthetic fatty acid gene encodes a polypeptide with epoxygenase or acetylenase or hydroxylase or desaturase activity, wherein said polypeptide either comprises an amino acid sequence which differs from a naturally-occurring epoxygenase or acetylenase or hydroxylase or desaturase enzyme, or said polypeptide exhibits catalytic properties which are different from a naturally-occurring epoxygenase or acetylenase or hydroxylase or desaturase enzyme or said polypeptide comprises a sequence of amino acids which are at least about 60% identical to a part of SEQ ID NO: 2 or 4 or 6 or 20 or homologue, analogue or derivative of said part.

Preferably, the synthetic fatty acid gene of the invention is derived from a Δ12 epoxygenase gene.

In one embodiment, the synthetic fatty acid gene of the invention encodes a fusion polypeptide in which the N-terminal and/or C-terminal amino acids of any one of SEQ ID

NOs: 2 or 4 or 6 or 20 are replaced, in-frame, by amino acid sequences of a different member of the same family.

In a particularly preferred embodiment, the N-terminal and/or C-terminal amino acids of SEQ ID NO: 2 or 4 or 6 or 20 are replaced by the corresponding regions of the acetylenase, desaturase or hydroxylase polypeptides set forth in Figure 2. More preferably, at least about 30 amino acid residues from the N-terminal and/or C-terminal regions of any one of SEQ ID NOs: 2 or 4 or 6 or 20 are replaced, in-frame, by the corresponding regions of the acetylenase, desaturase or hydroxylase polypeptides set forth in Figure 2.

In an alternative embodiment, the synthetic fatty acid gene of the invention encodes a fusion polypeptide in which the N-terminal and/or C-terminal amino acids of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6 or 20.

In a particularly preferred embodiment, the N-terminal and/or C-terminal amino acids of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6 or 20. Even more preferably, the fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase is selected from the list set forth in Figure 2.

Even still more preferably, at least about 30 amino acid residues from the N-terminal and/or C-terminal regions of a fatty acid acetylenase or fatty acid hydroxylase or fatty acid desaturase are replaced, in-frame, by the N-terminal and/or C-terminal region of any one of SEQ ID NOs: 2 or 4 or 6 or 20.

Accordingly, the present invention extends to any variants of the epoxygenase enzymes referred to herein, wherein said variants are derived from an epoxygenase polypeptide as described herein and exhibit demonstrable acetylenase or hydroxylase or desaturase activity, and either comprises an amino acid sequence which differs from a naturally-occurring acetylenase or hydroxylase or desaturase enzyme, or exhibit catalytic properties which are different from a naturally-occurring acetylenase or hydroxylase or desaturase enzyme, or comprise a sequence of amino acids which are at least about 60% identical to any one of SEQ ID NOs: 2 or 4 or 6 or 20.

As with other aspects of the invention, the variants described herein may be produced as recombinant polypeptides or in transgenic organisms, once the subject synthetic genes are introduced into a suitable host cell and expressed therein.

5 The recombinant polypeptides described herein or a homologue, analogue or derivative thereof, may also be immunologically active molecules.

A further aspect of the present invention provides an immunologically-interactive molecule which is capable of binding to a recombinant epoxygenase polypeptide of the invention.

10

Preferably, the recombinant epoxygenase polypeptide to which the immunologically-interactive molecule is capable of binding comprises a sequence of amino acids set forth in any one of SEQ ID NOs: 2, 4, 6, or 20, or a homologue, analogue or derivative thereof.

15 In one embodiment, the immunologically interactive molecule is an antibody molecule. The antibody molecule may be monoclonal or polyclonal. Monoclonal or polyclonal antibodies may be selected from naturally occurring antibodies to an epitope, or peptide fragment, or synthetic epoxygenase peptide derived from a recombinant gene product or may be specifically raised against a recombinant epoxygenase or a homologue, analogue or 20 derivative thereof.

Both polyclonal and monoclonal antibodies are obtainable by immunization with an appropriate gene product, or epitope, or peptide fragment of a gene product. Alternatively, fragments of antibodies may be used, such as Fab fragments. The present invention 25 extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies

The antibodies contemplated herein may be used for identifying genetic sequences which express related epoxygenase polypeptides encompassed by the embodiments described 30 herein.

The only requirement for successful detection of a related epoxygenase genetic sequence is that said genetic sequence is expressed to produce at least one epitope recognized by the antibody molecule. Preferably, for the purpose of obtaining expression to facilitate 35 detection, the related genetic sequence is placed operably behind a promoter sequence, for

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example the bacterial *lac* promoter. According to this preferred embodiment, the antibodies are employed to detect the presence of a plasmid or bacteriophage which expresses the related epoxygenase. Accordingly, the antibody molecules are also useful in purifying the plasmid or bacteriophage which expresses the related epoxygenase.

5

The subject antibody molecules may also be employed to purify the recombinant epoxygenase of the invention or a naturally-occurring equivalent or a homologue, analogue or derivative of same.

10

EXAMPLE 1

Characterization of epoxy fatty acids in *Euphorbia lagascae* and *Crepis spp.*

Seed from the wild species *Euphorbia lagascae* and from various *Crepis* species were screened by gas liquid chromatography for the presence of epoxy fatty acids. As shown in Table 3, *Euphorbia lagascae* contains very high levels of the epoxy fatty acid vernolic acid in its seed oil. Seeds from *Crepis palaestina* were shown to contain 61.4 weight % of vernolic acid and 0.71 weight % of the acetylenic fatty acid crepenynic acid of total fatty acids (Table 3).

15

20

TABLE 3

Fatty acid composition of lipids derived from seeds of *Crepis alpina*, *Crepis palaestina* and *Euphorbia lagascae*

Fatty acid	Relative distribution (weight %)[a]		
	Crepis alpina	*Crepis palaestina*	*Euphorbia*
Palmitic	3.9	5.1	4.3
Stearic	1.3	2.3	1.8
Oleic	1.8	6.3	22.0
Linoleic	14.0	23.0	10.0
Crepyninic	75.0	0.7	0
Vernolic	0	61.4	58.0
Other	4.0	1.2	3.9

[a] Calculated from the area % of total integrated peak areas in gas liquid chromatographic determination of methyl ester derivatives of the seed lipids

EXAMPLE 2

Biochemical characterization of linoleate Δ12-epoxygenases in *Euphorbia lagascae* and *Crepis palaestina*

The enzyme, linoleate Δ12-epoxygenase synthesizes vernolic acid from linoleic acid. Linoleate Δ12-epoxygenases derived from *Euphorbia lagascae* and *Crepis palaestina* are localized in the microsome. The enzymes from these species at least can remain active in membrane (microsome) fractions prepared from developing seeds.

Preparations of membranes from *Euphorbia lagascae* and assays of their epoxygenase activities were performed as described by Bafor *et al.* (1993) with incubations containing NADPH, unless otherwise indicated in Table 4. Lipid extraction, separation and methylation as well as GLC and radio-GLC separations were performed essentially as described by Kohn *et al.* (1994) and Bafor *et al.* (1993).

Preparations of membranes from *Crepis alpina* and *Crepis palaestina* were obtained as follows. *Crepis alpina* and *Crepis palaestina* plants were grown in green houses and seeds were harvested at the mid-stage of development (17-20 days after flowering). Cotyledons were squeezed out from their seed coats and homogenized with mortar and pestle in 0.1M phosphate buffer, pH 7.2 containing 0.33M sucrose, 4 mM NADH, 2 mM CoASH, 1 mg of bovine serum albumin/ml and 4,000 units of catalase/ml. The homogenate was centrifuged for 10 min at 18,000 x g and the resulting supernatant centrifuged for 60 min at 150,000 x g to obtain a microsome pellet.

Standard desaturase, acetylenase and epoxygenase assays with microsomal membranes from *Crepis* species were performed at 25°C with microsomal preparations equivalent to 0.2mg microsomal protein resuspended in fresh homogenization buffer and 10 nmol of either [1-^{14}C]18:1-CoA or [1-^{14}C]18:2-CoA (specific activity 85,000 d.p.m./nmol) in a total volume of 360µl. When NADPH was used as coreductant, the membranes were resuspended in homogenization buffer comprising NADPH in place of NADH.

Biochemical characterization of the microsomal linoleate Δ12-epoxygenase derived from *Euphorbia lagascae* and *Crepis palaestina* was carried out and data obtained were compared to the biochemical characteristics of oleate Δ12-desaturase and linoleate Δ12-acetylenase enzymes derived from microsomal preparations of *Crepis alpina* (Table 4).

As shown in Table 4, the *Crepis palaestina* linoleate Δ12-epoxygenase exhibits similar biochemical features to the linoleate Δ12-acetylenase and oleate Δ12-desaturase from *Crepis alpina,* in so far as all three enzymes require O_2, work equally well with either NADH or NADPH as the coreductants, and are inhibited by cyanide but not by carbon monoxide. Additionally, none of these enzymes are inhibited by monoclonal antibodies against cytochrome P450 reductase.

The data in Table 4 suggest that the *Crepis palaestina* linoleate Δ12-epoxygenase belongs to the same class of enzyme as the *Crepis alpina* microsomal oleate Δ12-desaturase and linoleate Δ12-acetylenase.

In contrast, the *Euphorbia lagascae* linoleate Δ12-epoxygenase requires NADPH as the coreductant, is not inhibited by cyanide, but is inhibited by carbon monoxide (Table 4). Additionally, the inventors have discovered that the *Euphorbia lagascae* linoleate Δ12-epoxygenase is inhibited by monoclonal antibodies raised against a cytochrome P450 reductase enzyme. These data suggest that the *Euphorbia lagascae* linoleate Δ12-epoxygenase belongs to the cytochrome P450 class of proteins and is therefore not related biochemically to the *Crepis palaestina* linoleate Δ12-epoxygenase.

TABLE 4

Comparison of the biochemical characteristics of epoxygenases, acetylenases and desaturases derived from *Crepis spp.* and *Euphorbia lagascae*

Treatment	Enzyme Activity (% of control)			
	C. alpina oleate Δ12-desaturase	*C. alpina* linoleate Δ12-acetylenase	*C. palaestina* linoleate Δ12-epoxygenase	*E. lagascae* linoleate Δ12-epoxygenase
Carbon monoxide	85	84	88	3
Anti-P450 reductase antibodies (C_5A_5)	96	91	94	33
KCN	16	0	35	92
minus NADH plus NADPH	95	73	94	100 (control)
minus NADPH plus NADH	100 (control)	100 (control)	100 (control)	11

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5

EXAMPLE 3

Strategy for cloning *Crepis palaestina* epoxygenase genes

Cloning of the *Crepis palaestina* epoxygenase genes relied on the characteristics of the *C. palaestina* and *C. alpina* enzymes described in the preceding Examples.

10

In particular, poly (A)+ RNA was isolated from developing seeds of *Crepis palaestina* using a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology) and used to synthesize an oligosaccharide d(T)-primed double stranded cDNA. The double stranded cDNA was ligated to *Eco*RI/*Not*I adaptors (Pharmacia Biotechnology) and a cDNA library
15 was constructed using the ZAP-cDNA Gigapack cloning kit (Stratagene).

Single-stranded cDNA was prepared from RNA derived from the developing seeds of *Crepis alpina,* using standard procedures. A PCR fragment, designated as D12V (SEQ ID

NO: 7), was obtained by amplifying the single-stranded cDNA using primers derived from the deduced amino acid sequences of plant mixed-function monooxygenases.

The D12V fragment was subsequently random-labeled and used to screen the *Crepis* 5 *palaestina* cDNA library *supra* on Hybond N+ membrane filters from Amersham as prescribed by the manufacturer using standard hybridization conditions. This approach resulted in the purification of a recombinant bacteriophage, designated Cpa12.

The nucleotide sequence of the Cpa12 cDNA was determined and is set forth in SEQ ID 10 NO: 1.

The Cpa12 cDNA appeared to be full-length. A schematic representation of an expression vector comprising the Cpa12 cDNA is presented in Figure 1. The gene construct set forth therein is designed for introduction into plant material for the production of a transgenic 15 plant which expresses the subject epoxygenase. Those skilled in the art will recognise that similar expression vectors may be produced, without undue experimentation, and used for the production of transgenic plants which express any of the genetic sequences of the instant invention, by replacing the Cpal2 cDNA with another structural gene sequence.

20 As shown in Figure 2, the nucleotide sequence of the Crep1 cDNA encoded a polypeptide which was closely related at the amino acid level, at least, to an acetylenase enzyme of *C. alpina* (Bafor *et al.* 1997; International Patent Application No. PCT/SE97/00247).

The 1.4 kb insert from pCpal2 was sequenced (SEQ ID NO. 1) and shown to comprise an 25 open reading frame which encodes a polypeptide of 374 amino acids in length. The deduced amino acid sequence of Cpal2 showed 81% identity and 92% similarity to the Δ12-acetylenase from *Crepis alpina* and approximately 60% identity and 80% similarity with plant microsomal Δ12-desaturase proteins (Figure 2). However, the polypeptide encoded by Cpal2 comprised significant differences in amino acid sequence compared to non-30 epoxygenase enzymes. In particular, the Cpa12 has a deletion of six contiguous amino acids in the 5'-terminal region compared to all the microsomal Δ12 desaturases, and a deletion of two contiguous amino acids in the 3'-terminal region compared to the Crep1 Δ12 acetylenase (Figure 2).

Although membrane-bound fatty acid desaturase genes show limited sequence homologies, they all contain three regions of conserved histidine-rich motifs as follows:

(i) His-$(Xaa)_{3-4}$-His (SEQ ID NO: 21 and SEQ ID NO: 22);

(ii) His-$(Xaa)_{2-3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24); and

5 (iii) His-$(Xaa)_{2-3}$-His-His (SEQ ID NO: 23 and SEQ ID NO: 24),

wherein His designates histidine, Xaa designates any naturally-occurring amino acid residue as set forth in Table 1 herein, the integer $(Xaa)_{3-4}$ refers to a sequence of amino acids comprising three or four repeats of Xaa, and the integer $(Xaa)_{2-3}$ refers to a sequence of amino acids comprising two or three repeats of Xaa. These histidine-rich regions are 10 suggested to be a part of the active center of the enzyme (Shanklin *et al.*, 1994).

The amino acid sequence encoded by the Cpal2 cDNA comprises three histidine-rich motifs similar, but not identical, to the histidine-rich motifs of the Δ12-desaturase enzymes. These data suggest that the Cpa12 cDNA encodes an enzyme which belongs to the mixed 15 function monooxygenase class of enzymes.

The analysis of fatty acids presented in Example 1 *supra* indicated that vernolic acid was at least present in the seeds of *Crepis palaestina*. This enzyme may in fact be present exclusively in the seeds of *C. palaestina*. The expression of the Cpal2 gene was examined 20 using the 3' untranslated region of the Cpal2 cDNA clone as a hybridization probe on northern blots of mRNA derived from developing seeds and leaves of *C. palaestina*. As shown in Figure 3, the Cpal2 gene was highly expressed in developing seeds but no expression could be detected in leaves. These data are consistent with the enzyme activity profile of *C. palaestina* linoleate Δ12-epoxygenase in these tissues.

25

EXAMPLE 4

Demonstration of epoxygenase activity for the *C. palaestina* clones

Confirmation that the cDNA clones of *C. palaestina* encode an epoxygenase was obtained by transforming *Arabidopsis thaliana,* with each individual candidate clone. The transformed 30 tissue was examined for the presence of epoxy fatty acids that *A. thaliana* would not otherwise produce. Additionally, the level of hydroxy fatty acids was determined, as such fatty acids can be formed from the metabolism of an epoxy fatty acid, by the action of endogenous *A. thaliana* epoxide hydrolases (Blee and Schuber, 1990).

The epoxygenase cDNA comprising SEQ ID NO: 1 was cloned into the Binary vector construct set forth in Figure 4. Briefly, the cDNA sequence was sub-cloned from the pCpal2 plasmid (Figure 1) into the binary plasmid, by digesting pCpal2 with *Eco*RI and end-filling the restriction fragment using T4 DNA polymerase enzyme. The Binary vector (Figure 4) was made linear using *Bam*HI and also end-filled using T4 DNA polymerase. For the end-filling reactions, 1µg of cDNA insert or linearized Binary vector DNA was resuspended in 50µl of T4 DNA polymerase buffer (33mM Tris-acetate pH 7.9, 66mM potassium acetate, 10mM magnesium acetate and 5mM DDT) supplemented with 100mM of each dNTP and 0.1mg/ml BSA and 3 units of T4 DNA polymerase, and incubated for 6 min incubation at 37°C. The reaction was stopped by heating at 75□C for 10mins. The blunt-ended cDNA and Binary vector DNA were ligated using T4 DNA ligase and standard ligation conditions as recommended by Promega. Clones were selected in which the SEQ ID NO: 1 sequence was inserted behind the napin promoter, in the sense orientation, thereby allowing for expression of the epoxygenase polypeptide. The Binary plasmid harboring SEQ ID NO: 1, in the sense orientation, operably under control of the truncated napin promoter, is represented schematically in Figure 5.

The Binary plasmid set forth in Figure 5 was transformed into *Agrobacterium* strain AGLI using electroporation and used to transform *Arabidopsis thaliana*. Transgenic *A. thaliana* plants were obtained according to the method described by Valvekens *et al.* (1988) and Dolferus *et al.* (1994).

Transgenic plants and untransformed (i.e. control) plants were grown to maturity. Mature seed of each plant was analyzed for fatty acid composition by standard techniques. Primary transformant (T_0) plants were established and T1 seed was harvested from each plant and analyzed for fatty acid composition by gas chromatography. Twelve T_0 plants were shown to contain vernolic acid in their T1 seed lipids at concentrations ranging from 0.9% to 15.8% of total fatty acids, while untransformed control plants contained no vernolic acid (Table 5). The highest-expressing plant line was Cpal-17, for which the GLC elution profiles (from packed column and capillary column analysis) is presented in Figure 6. The GLC elution profile from packed column for the untransformed control is also shown in Figure 6.

TABLE 5

Vernolic acid levels in transgenic *A. thaliana* lines expressing SEQ ID NO: 1

T_0 Plant No.	Vernolic acid (weight % of total seed fatty acids)
Cpal-4	1.4
Cpal-5	1.1
Cpal-8	2.7
Cpal-9	0.9
Cpal-13	0.9
Cpal-15	1.1
Cpal-17	15.8
Cpal-21	1.3
Cpal-23	1.4
Cpal-24	1.0
Cpal-25	1.2
Cpal-26	1.1
untransformed control line	0.0

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5

Alternatively, or in addition, putative fatty acid epoxygenase sequences described herein are each transformed into *Linum usitatissimum* (flax) and *Arabidopsis thaliana* under the control of the napin seed-specific promoter. Transgenic flax and *Arabidopsis thaliana* plants are examined for presence of epoxy fatty acids in developing seed oils. Previous
10 work has shown that if epoxy fatty acids are fed to developing flax embryos they are incorporated into triglycerides (Example 10).

Alternatively, yeast are also transformed with the epoxygenase clones of the invention and assayed for production of epoxy fatty acids.

15

EXAMPLE 6

Mass spectroscopy confirmation of epoxy fatty acids in T_1 *Arabidopsis* seed borne on primary T_0 transgenic plants

5 Gas chromatography of methyl esters prepared from seed lipids of T1 seed of *Cpal2*-transformed *Arabidopsis thaliana* plants (Example 5) revealed the presence of two additional fatty acids compared to the untransformed controls. The first of these compounds had a retention time equivalent to that of a vernolic acid standard. The second compound had a longer retention time and was putatively identified as 12,13-10 epoxy-9,15-octadecadienoic acid, an expected derivative of vernolic acid, resulting from desaturation at the Δ15 position by the endogenous *Arabidopsis thaliana* Δ15-desaturase.

Confirmation of the exact identity of the two peaks was obtained by mass spectroscopy of diols which were prepared from the epoxy fatty acid fraction derived from *Cpal2*-15 transformed plants. The diols were converted further to trimethylsilyl ethers and analyzed by GC-MS DB23 on a fused silica capillary column (Hewlett-Packard 5890 II GC coupled to a Hewlett Packard 5989A MS working in electron impact at 70eV15). The total ion chromatogram showed two peaks as follows:

(i) The first eluting peak had prominent ions of mass 73, 172, 275, and 299, 20 indicating that the epoxy group was positioned at C-12 of a C18 fatty acid and that a double bond occurred between the epoxy group and the carboxyl terminus. This mass spectra was identical to the spectra of a trimethylsilyl ether derivative of diols prepared from pure vernolic acid (12,13-epoxy-9-octadecenoic acid); and

25 (ii) the second eluting peak had prominent ions of mass 73, 171, 273, and 299, indicating the presence of two double bonds and an epoxy group positioned at C-12 of a C18 fatty acid, consistent with the mass spectrum for 12,13-epoxy-9,15-octadecadienoic acid.

30

EXAMPLE 7

Fatty acid analysis of *Cpal2* transgenic *Arabidopsis* plants

The T1 seed derived from transformed *Arabidopsis thaliana* plants expressing the Cpal2 cDNA clone under control of the napin promoter was germinated and T1 plants were

established from five T_0 lines (Nos. 4, 8, 13, 17 & 21 in Table 5). The T2 seed was harvested from each T1 plant and analyzed for fatty acid composition. The progeny of transformant Nos. 4, 8, 13 and 21 (Table 5) segregated as expected for presence of vernolic acid, with those plants containing vernolic acid ranging up to 3.1% (Table 6).

5

All T1 plants that contained vernolic acid (i.e. epoxy 18:1 in Table 6) also contained 12,13-epoxy-9,15-octadecadienoic acid (i.e. epoxy 18:2 in Table 6; see also Figure 7), indicating that some of the vernolic acid synthesized by the *Cpal2* epoxygenase was subsequently desaturated by the endogenous Δ15-desaturase.

10

TABLE 6

Fatty acid composition of selfed seeds borne on T_1 plants derived from five primary *Cpal2* transformants of *Arabidopsis thaliana*

Plant No.	Fatty Acid										
	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
4-1	8.3	3.9	15.5	23.9	20.6	2.8	16.5	1.7	1.6	-	-
4-2	7.6	4.1	20.3	17.8	18.0	3.4	19.7	1.8	2.0	0.82	0.63
4-3	8.4	4.3	26.0	13.5	16.1	2.8	19.0	1.8	1.6	2.03	0.72
4-4	7.6	4.0	25.2	14.3	16.0	2.8	19.8	2.1	1.7	1.99	0.92
4-5	7.2	3.6	15.6	23.1	19.9	3.1	19.7	1.6	2.1	-	-
4-6	7.0	3.7	19.2	17.8	18.4	3.2	20.3	1.9	2.1	0.87	0.33
4-8	7.4	3.9	16.0	23.6	20.1	3.1	18.7	1.6	1.8	-	-
4-9	7.6	4.0	24.8	13.4	15.9	2.8	20.4	2.3	1.8	2.30	1.07
4-10	7.6	4.2	24.0	13.5	16.2	3.1	20.4	1.9	1.8	1.97	0.83
4-11	7.4	3.9	15.0	23.2	20.4	3.3	18.8	1.7	2.0	-	-
4-12	8.7	4.0	20.7	17.0	17.5	2.6	17.2	1.7	1.5	1.38	0.74
4-13	7.2	4.1	21.9	16.4	17.7	3.2	21.0	1.7	1.9	1.14	0.45
8-1	8.1	3.9	26.1	15.0	16.0	2.6	19.5	2.0	1.6	1.79	0.82
8-3	8.7	4.2	31.6	11.5	14.0	2.2	18.5	1.9	1.4	2.38	1.13
8-4	8.5	4.1	27.2	15.1	16.1	2.5	18.9	1.8	1.4	1.70	0.84
8-5	9.1	4.2	27.7	14.7	16.2	2.4	18.3	1.7	1.5	1.70	0.82
8-6	9.8	4.0	26.0	17.2	17.2	2.3	16.9	1.6	1.2	1.36	0.71
8-7	10.0	3.5	15.2	25.3	22.3	2.3	14.4	1.7	1.7	-	-
8-8	8.4	4.3	32.2	10.7	13.3	2.5	20.3	1.6	1.5	1.92	0.82
8-9	9.8	3.6	15.9	25.3	22.0	2.4	14.5	1.6	1.3	-	-
8-10	7.5	3.9	24.4	15.9	15.8	2.8	20.2	2.2	1.8	1.70	0.82
8-11	7.6	3.8	15.4	23.6	19.8	2.9	19.4	1.5	1.8	-	-
8-12	9.4	3.7	24.2	16.7	16.7	2.2	17.6	0.9	1.2	1.46	0.65
8-13	10.3	4.3	25.3	17.1	17.9	2.2	16.0	1.8	1.3	1.48	0.73
13-1	7.0	4.3	33.3	8.1	11.1	2.7	23.1	1.7	1.6	2.42	1.26
13-2	7.2	4.3	30.4	9.6	12.7	2.8	22.0	1.8	1.6	2.48	1.37
13-3	7.6	3.9	15.6	23.6	19.7	3.0	19.1	1.7	1.8	-	-
13-4	7.7	4.0	15.2	22.5	19.3	3.1	18.0	1.6	1.7	-	-
13-5	8.0	4.2	16.3	22.2	17.5	4.4	19.4	2.0	2.0	-	-
13-6	7.9	4.4	25.7	14.7	15.8	2.9	21.2	1.6	1.7	1.56	0.63

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Plant No.	Fatty Acid										
	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
13-7	7.9	4.0	16.0	23.3	19.6	3.0	19.1	1.6	1.8	-	-
13-9	8.0	4.0	16.1	23.6	20.0	2.9	18.7	1.6	1.6	-	-
13-10	8.7	4.2	34.6	9.6	12.5	2.2	19.1	1.5	1.2	2.21	1.01
13-11	8.7	4.0	17.6	24.3	18.9	2.8	17.1	1.6	1.4	-	-
13-12	8.9	4.2	26.4	14.6	16.0	2.5	17.5	1.6	1.2	1.62	0.74
13-13	9.0	4.4	27.9	14.4	15.3	2.5	18.9	1.5	1.4	1.30	0.77
13-14	9.2	4.2	17.2	23.8	18.8	2.7	17.9	1.7	1.5	-	-
13-15	8.4	4.2	19.7	20.9	18.6	2.7	17.7	1.4	1.5	0.40	0.16
13-16	8.2	4.3	23.0	17.1	17.3	2.8	19.3	1.5	1.5	0.97	0.42
13-17	8.3	4.1	15.7	23.9	19.9	2.8	17.6	1.6	1.9	-	-
17-1	7.6	4.1	15.8	23.7	19.6	2.6	20.3	1.7	1.7	-	-
17-2	8.3	4.1	16.4	24.4	20.1	2.3	16.8	1.5	1.4	-	-
17-3	8.1	4.1	16.4	24.3	20.0	2.5	17.6	1.6	1.4	-	-
21-1	8.1	4.3	26.9	14.5	15.0	2.9	19.9	1.5	1.5	1.64	0.63
21-2	8.2	4.0	27.9	11.8	13.2	2.5	19.8	1.7	1.5	2.18	0.91
21-3	8.8	3.7	16.4	24.4	20.6	2.5	17.3	1.7	1.4	-	-
21-4	7.9	3.9	19.6	19.8	17.8	2.7	18.7	1.7	1.7	0.66	0.46
21-5	7.2	4.2	26.5	12.9	14.4	3.0	21.5	0.9	1.8	1.78	0.84
21-6	8.3	4.2	27.4	13.9	15.4	2.6	19.9	1.7	1.5	1.66	0.65
21-7	7.2	4.2	26.8	13.5	13.4	3.0	21.9	1.7	1.8	1.74	0.80
21-8	7.4	3.8	16.3	23.6	19.4	3.2	19.2	1.7	1.9	-	-
21-9	7.2	4.0	28.1	11.8	13.5	3.0	22.5	1.9	1.9	2.15	1.05
21-10	7.2	4.2	26.1	13.8	14.6	3.0	22.3	1.7	1.8	1.64	0.82
21-11	7.1	4.2	29.2	11.5	12.7	3.0	22.5	1.8	1.8	2.20	1.09
21-12	7.2	4.1	26.2	13.6	14.2	3.1	22.4	1.8	1.9	1.71	0.80
21-13	7.1	4.3	33.7	7.1	10.0	2.7	24.1	2.0	1.8	3.05	1.47
21-14	7.4	3.7	16.9	21.9	19.6	3.1	19.2	1.8	2.0	0.29	tr
21-15	7.7	3.6	15.6	24.3	20.2	2.9	18.1	1.8	1.8	-	-

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EXAMPLE 8

Fatty acid analysis of Cpal2 transgenic Linola plants

The binary plasmid construct described above comprising the Cpal2 cDNA clone (Figure 5) was transformed into *Agrobacterium tumefaciens* strain AGL1, using electroporation. The transformed *A. tumefaciens* was used to infect *Linum usitatissimum* var. Eyre explants as described by Lawrence *et al* (1989), except that MS media was used as the basal medium for the induction of roots on regenerated shoot material.

Two primary Linola transformants (T_0 plants) designated AP20 and AP21 were confirmed as being transgenic by PCR using primers directed against the Cpal2 gene and by showing that these plants were kanamycin resistant. Ten T1 seeds from each plant were analyzed individually for fatty acid composition using standard techniques.

As shown in Table 7, seed from AP20 segregated into 3 classes, comprised of three seeds with no vernolic acid, two having greater than 0.7% vernolic acid, and five having intermediate levels (0.13-0.47%) of vernolic acid.

Similarly, seeds from AP21 segregated into 3 classes comprised of five seeds having no vernolic acid, two having greater than 0.25% vernolic acid and three having an intermediate level (0.09-0.14%) of vernolic acid (Table 8).

Thus, a total of twelve seeds were obtained which contained vernolic acid. Eight of the twelve AP20 and AP21 seeds containing vernolic acid also contained 12,13-epoxy-9,15-octadecadienoic acid.

TABLE 7

Fatty acid composition of 10 individual T1 seeds from

Linola *Cpal2* primary transformant AP20

T_1 seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.4	3.6	17.8	68.1	2.0	0.2	-	0.6	-	-	-
2	6.0	3.5	25.4	60.8	1.4	0.2	0.2	-	-	0.70	0.23
3	6.0	3.9	20.4	64.6	2.1	0.3	0.6	-	-	-	-
4	6.3	3.5	28.3	57.3	1.3	0.2	0.2	1.4	-	0.34	0.28
5	5.2	4.8	24.9	61.2	1.6	0.3	0.2	0.1	-	0.37	-
6	5.8	4.1	23.3	63.1	1.9	0.2	0.2	0.2	-	0.47	-
7	5.9	4.3	21.7	64.1	2.2	0.2	0.2	0.2	-	0.13	0.12
8	5.9	3.3	22.3	65.2	2.0	0.2	0.2	0.1	0.2	-	-
9	5.6	4.0	25.2	61.4	1.7	0.2	0.2	0.1	-	0.84	-
10	6.2	4.4	27.4	57.9	1.7	0.2	0.2	0.2	-	0.54	-

5

TABLE 8

Fatty acid composition of 10 individual T1 seeds from

Linola *Cpal2* primary transformant AP21

T_1 seed	Non-epoxy fatty acids									Epoxy fatty acids	
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1	18:2
1	6.1	4.2	35.2	50.8	1.3	-	-	-	2.0	-	-
2	5.7	5.0	32.9	53.3	1.4	0.2	0.2	0.2		0.14	0.21
3	5.9	4.0	35.1	50.8	1.3	0.2	0.2	0.1	1.5	-	-
4	7.5	4.1	38.8	45.5	1.2	0.2	0.3	-	1.7	-	-
5	5.8	5.0	28.8	57.3	1.3	0.2	0.2	0.1	-	0.37	0.06
6	5.8	5.0	44.1	41.4	1.4	0.2	0.2	0.2	-	-	-
7	6.5	4.5	27.9	58.6	1.3	0.2	0.1	0.1	-	-	-
8	6.9	4.6	37.6	48.1	1.2	-	-	-	-	0.10	0.19
9	6.2	4.7	33.7	52.1	1.3	0.2	0.2	0.2	-	0.09	0.07
10	6.1	4.8	29.7	56.6	1.3	0.2	0.2	0.1	-	0.25	0.04

10

Four T1 plants were established from the kanamycin-resistant seedlings of AP20. All four plants were subsequently shown to produce vernolic acid in their T2 seed (Table 9). Levels of 18:2 epoxy fatty acids were not analyzed in these T2 seed.

TABLE 9

Fatty acid composition of T2 seeds from Linola *Cpal2* T1 progeny of AP20

T_2 seed	Non-epoxy fatty acids									epoxy fatty acid
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	18:1
A	3.4	3.0	27.4	65.5	0.6	na	na	na	na	0.06
B	3.5	3.1	30.2	62.6	0.6	na	na	na	na	0.07
C	3.6	2.7	33.3	59.8	0.6	na	na	na	na	0.07
D	3.4	3.1	28.2	64.6	0.6	na	na	na	na	0.11

na. = not analyzed

5

EXAMPLE 9

Producing epoxy fatty acids in transgenic organisms

10 Production of an oil rich in vernolic acid was achieved by transforming the epoxygenase gene described herein, in particular SEQ ID NO: 1, into *Arabidopsis thaliana*, as described in the preceding Examples. As shown in Table 5, transgenic *A. thaliana* lines expressing SEQ ID NO: 1 produce high levels of vernolic acid in their seeds relative to other fatty acids. In particular, in one transgenic line (Cpal-17), the vernolic acid produced is as much
15 as 15.2% (w/w) of total seed fatty acid content.

Production of an oil rich in vernolic acid is also achieved by transforming the epoxygenase gene described herein, in any one of SEQ ID NOs: 1, 3, 5, or 19 and preferably any one of SEQ ID NOs:1 or 3 or 5 or 19, into any oil accumulating organism that normally has very
20 high levels of linoleic acid and minimal other competing enzyme activities capable of utilizing linoleic acid as a substrate. The genetic sequences of the invention are placed operably under the control of a promoter which produces high-level expression in oilseed, for example the napin seed-specific promoter.

In one alternative approach to the transformation of *A. thaliana*, high-linoleic genotypes of flax, sunflower, corn or safflower are transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

5

Alternatively, Linola™ (= low linolenic acid) flax is transformed with the epoxygenase of the invention. High levels of vernolic acid are produced by the transgenic Linola™ flax plants during seed oil synthesis, when the epoxygenase gene is expressed at high levels.

10 Additionally, the inventors have shown that labeled vernolic acid fed to developing flax seeds is not degraded but is incorporated into storage lipids at all three positions of the triglyceride molecule (see Example 10). Consistent with these data, high levels of vernolic acid synthesized by the introduced epoxygenase are readily deposited into the seed oil triglycerides of this species.

15

EXAMPLE 10

Incorporation of oleic acid and vernolic acid into the lipids of developing linseed cotyledons

20

Detached developing linseed cotyledons (six pairs in each incubation, duplicate incubations) at mid stage of seed development (20 days after flowering) were incubated with 10 nmol of the ammonium salts of either [1-^{14}C]vernolic acid (specific activity 3000 d.p.m./nmol) or [1-^{14}C]oleic acid (specific activity 5000 d.p.m./nmol) in 0.2 ml phosphate
25 buffer pH 7.2 for 30 min at 30□C. The cotyledons were then rinsed three times with 1 ml of distilled water and either extracted immediately in an Ultra Turrax according to Bligh and Dyer (1959) or incubated further in 0.5 m. 0.1 M phosphate buffer pH 7.2 for 90 or 270 min before extraction. An aliquot of the lipids in the chloroform phase was methylated and separated on silica gel TLC plates in n-hexane/diethylether/acetic acid (85:15:1). The
30 rest of the lipids in the chloroform phase of each sample were applied on two separate silica gel TLC plates and the plates were developed in chloroform/methanol/acetic acid/water (85:15:10:3.5 by vol) for polar lipids separation and in n-hexane/diethylether/acetic acid (60:40:1.5) for neutral lipid separation. Lipid areas with

migration corresponding to authentic standards were removed and radioactivity in each lipid were quantified by liquid scintillation counting.

The recovery of ^{14}C-label in the chloroform phase is depicted in Figure 8. Somewhat
5 more than half of added radioactivity from both [^{14}C]oleic acid and [^{14}C]vernolic acid was taken up by the cotyledons and recovered as lipophilic substances after the 30 min pulse labeling. This quantity remained virtually unchanged during the further 270 min of incubation with both substrates. Separation of radioactive methyl esters of the lipids showed that most of the radioactivity (92%) from [^{14}C]vernolic acid feeding experiments
10 resided in compounds with the same migration as methyl-vernoleate indicating that the epoxy group remained intact in the linseed cotyledons throughout the 270 min incubation.

About 28% of the activity from [^{14}C]vernolic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity
15 decreased to only 5% at 300 min of incubation (Figure 9).

About 22% of the activity from [^{14}C]oleic acid feeding which was present in the chloroform phase resided in phosphatidylcholine after 30 min and the radioactivity decreased to about 11% at 300 min of incubation (Figure 9).

20

About 32% of the activity from [^{14}C]vernolic acid feeding which was present in the chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to over 60% at 300 min of incubation (Figure 10). The diacylglycerols contained some 24% of the activity in the [14C]vernolic acid feeding experiments and this quantity remained
25 rather constant over the incubation periods.

About 5% of the activity from [^{14}C]oleic acid feeding which was present in the chloroform phase resided in triacylglycerols after 30 min and the radioactivity increased to 18% at 300 min of incubation (Figure 10). The diacylglycerols contained some 19% of the activity
30 after 30 min in the [^{14}C]oleic acid feeding experiments and this quantity remained rather constant over the incubation periods.

The above experiment shows that linseed cotyledons do not metabolize the epoxy group of vernolic acid to any great extent. Further it shows that linseed cotyledons possess

mechanisms to efficiently remove vernolic acid from membrane lipids and incorporate them into triacylglycerols.

5 EXAMPLE 11

Cloning of Δ12-epoxygenase genes from an unidentified *Crepis* species

Homologues of the Cpal2 Δ12-epoxygenase gene were obtained from species other than *C. palaestina* which are rich in epoxy fatty acids, by cloning the members of the gene family of Δ12 mixed function monooxygenases that are highly expressed in developing 10 seeds and comparing their amino acid sequence to those of known Δ12-desaturase and Δ12-epoxygenase sequences.

Such genes were cloned either by screening developing seed cDNA libraries with genetic probes based on either the Cpal2 gene (SEQ ID NO: 1) or the D12V fragment (SEQ ID 15 NO: 7), or by amplifying PCR fragments using primers designed against conserved sequences of the plant Δ12 mixed function monooxygenases, as described herein. Putative Δ12-epoxygenase sequences show greater overall sequence identity to the Δ12-epoxygenase sequences disclosed herein, than to the known Δ12-desaturase sequences.

20 In one example of this approach, a full-length Δ12-epoxygenase-like sequence was obtained from an unidentified *Crepis sp.* containing high levels of vernolic acid in its seed oils and known not to be *Crepis palaestina*. Poly(A)+ RNA was isolated from developing seeds of this *Crepis sp.* using a QuickPrep Micro mRNA purification kit (Pharmacia Biotechnology) and used to synthesize an oligosaccharide d(T)-primed double-stranded 25 cDNA. The double stranded cDNA thus obtained was then ligated to *Eco*R1/ *Not*I adaptors (Pharmacia Biotechnology) and a cDNA library was constructed using the ZAP-cDNA Gigapack cloning kit (Stratagene). The cDNA library on Hybond N+ membrane filters (Amersham) was screened with the random-labeled D12V fragment (SEQ ID NO: 7) derived from *Crepis alpina* as prescribed by the manufacturer, using standard 30 hybridization conditions. This resulted in the purification of a recombinant bacteriophage designated CrepX.

The nucleotide sequence of the CrepX cDNA was determined and is set forth in SEQ ID NO: 3. The deduced amino acid sequence of CrepX (SEQ ID NO: 4) comprises a 374

amino acid protein having 97% identity to the Cpal2 Δ12-epoxygenase sequence, but only 57% identity to the *Arabidopsis thaliana* L26296 Δ12-desaturase sequence. This clearly demonstrates the presence of a gene in another *Crepis sp.* having high vernolic acid content, which gene is highly homologous to the Cpal2 Δ12-epoxygenase gene and is
5 clearly not a desaturase gene.

EXAMPLE 12

Cloning of Δ12-epoxygenase genes from *Vernonia galamensis*

10 Following the general strategy outlined in the preceding example, a homologue of the Cpal2 Δ12-epoxygenase gene was also obtained from *Vernonia galamensis*, containing high levels of vernolic acid in its seeds.

A partial Δ12-epoxygenase-like sequence was obtained from *V. galamensis*, by preparing
15 first strand cDNA templates using total RNA from developing seeds as a template. A PCR fragment (550 nucleotides in length), designated as Vgal1, was obtained by amplifying the single-stranded cDNA, using primers derived from the deduced amino acid sequence of plant mixed function monooxygenases. The nucleotide sequence of the amplified DNA was determined using standard procedures and is set forth in SEQ ID NO: 5.
20

Alignment of the deduced amino acid sequence of the Vgal1 PCR fragment (SEQ ID NO: 6) with the full sequence of Cpal2 Δ12-epoxygenase and the *Arabidopsis thaliana* L26296 Δ12-desaturase (Figure 2) demonstrates that the amplified Vgal1 sequence encodes an amino acid sequence corresponding to the region spanning amino acid residues 103-285
25 of the Cpal2 polypeptide. Within this region, the Vgal1 sequence showed greater amino acid identity with the Cpal2 Δ12-epoxygenase sequence (67%) than with the *A. thaliana* Δ12-desaturase sequence (60%), suggesting that the amplified DNA corresponds to an epoxygenase rather than a desaturase sequence.

30 The corresponding full-length Δ12-epoxygenase sequence was obtained from *V. galamensis,* and the nucleotide sequence of the full-length clone determined (SEQ ID NO: 19). The deduced amino acid sequence of the full-length Vgal Δ12-epoxygenase polypeptide (SEQ ID NO: 20) comprises 384 amino acids comprising all three conserved

mixed function monooxygenase consensus sequences for epoxygenases as set forth in SEQ ID NOs: 15, 16, and 18 (see Figure 2).

5

EXAMPLE 13

Demonstration of epoxygenase activity for the *V. galamensis* clone

Confirmation that the full-length cDNA clone of *V. galamensis* encodes an epoxygenase is obtained by transforming *Arabidopsis thaliana* with a binary vector comprising the isolated cDNA clone in the sense orientation and in operable connection with a promoter as
10 described in the preceding examples. Transformed tissue is examined for the presence of epoxy fatty acids that *A. thaliana* would not otherwise produce. Additionally, the level of hydroxy fatty acids is determined, as such fatty acids can be formed from the metabolism of an epoxy fatty acid, by the action of endogenous *A. thaliana* epoxide hydrolases (Blee and Schuber, 1990).

15

The *V. galamensis* cDNA (SEQ ID NO: 19) was cloned into a binary vector construct, such as that shown in Figure 4, essentially as described in the preceding examples. The Binary plasmid harboring SEQ ID NO: 19 was transformed into *Agrobacterium* strain AGLI using electroporation and used to transform *A. thaliana*. Transgenic *A. thaliana* plants
20 were obtained according to the method described by Valvekens *et al.* (1988) and Dolferus *et al.* (1994).

Transgenic plants and untransformed (i.e. control) plants are grown to maturity. Mature seed of each plant are analyzed for fatty acid composition by standard techniques.
25 Primary transformant (T_0) plants are established and T1 seed are harvested from each plant and analyzed for their fatty acid composition by gas chromatography. T_0 plants are shown to contain higher levels of epoxy fatty acids in their T1 seed lipids than the seeds of untransformed control plants.

30 Gas chromatography of methyl esters prepared from seed lipids of T1 seed of *Vgal* transformed *Arabidopsis thaliana* plants is performed to show the presence of additional fatty acids compared to the untransformed controls. The retention time of these compounds permits their identification as epoxy fatty acids, and/or derivatives of epoxy

fatty acids that are produced by the action of endogenous desaturase enzymes on the epoxy fatty acids.

Confirmation of the exact identity of the epoxy fatty acid products and derivatives thereof
5 is obtained by mass spectroscopy of the diols from the epoxy fatty acid fraction of transformed plants. The diols are converted further to trimethylsilyl ethers and analyzed by GC-MS DB23 on a fused silica capillary column (Hewlett-Packard 5890 II GC coupled to a Hewlett Packard 5989A MS working in electron impact at 70eV15).

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